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TRANSMITTAL LETTER
SUPPLEMENTAL APPEAL BRIEF

Applicant	:	Paul P. Latta
App. No	:	10/660,924
Filed	:	September 12, 2003
For	:	PREVENTION OF DIABETES THROUGH INDUCTION OF IMMUNOLOGICAL TOLERANCE
Examiner	:	Balyavskyi, Michail
Art Unit	:	1644

CERTIFICATE OF EXPRESS MAILING

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July 19, 2006
(Date)

Daniel E. Altman, Reg. No. 34,115

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith for filing in the above-identified application are the following enclosures:

(X) Copy of Notification of Non-Compliant Appeal Brief, mailed June 19, 2006.

(X) Supplemental Appeal Brief in 14 pages.

(X) Specification as filed.

(X) Declaration of David Scharp, signed November 11, 2003 and filed with Appendix A.

(X) Office Action, mailed December 16, 2004.

(X) Response to Office Action, filed April 29, 2005.

(X) Second Declaration of David Scharp, signed April 28, 2005 and filed April 29, 2005 (with Exhibit A, Appendix 1 and Appendix 2).

(X) Appendix 1 -http://www.niaid.nih.gov/dait/NODmice.htm printout.

(X) Appendix 2 - Hanninen et al. 2003 "Development of new strategies to prevent type I diabetes: the role of animal models" *Annals of Medicine* **35**:546-563.

(X) Bonifacio et al. 1995 "Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity", *Diabetologia* **38**:816-22, filed April 29, 2005.

(X) Lee et al. 1995 "Relationships among 64k autoantibodies, pancreatic beta-cell function, HLA-DR antigens and HLA-DQ genes in patients with insulin-dependent diabetes mellitus in Korea", *Korean J. Intern. Med.* **10**:1-9, abstract only, filed April 29, 2005.

(X) Bingley et al. 1994 "Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives", *Diabetes* **43**:1304-10, filed April 29, 2005.

(X) Zimmet et al. 1994 "Autoantibodies to glutamic acid decarboxylase and insulin in islet cell antibody positive presymptomatic type 1 diabetes mellitus: frequency and segregation by age and gender", *Diabet Med.* **11**:866-71, filed April 29, 2005.

(X) Christie et al. 1994 "Antibodies to islet 37k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity", *Diabetes* **43**:1254-9, filed April 29, 2005.

(X) Tuomilehto et al. 1994 "Antibodies to glutamic acid decarboxylase as predictors of insulin-dependent diabetes mellitus before clinical onset of disease", *Lancet* **343**:1383-5, filed April 29, 2005.

(X) Zimmet et al. 1994 "Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency", *Diabet Med.* **11**:299-303, filed April 29, 2005.

(X) Final Office Action, mailed May 18, 2005.

(X) Return prepaid postcard.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Dated: July 19, 2006

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2762883:VT071806

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/660,924	09/12/2003	Paul P. Latta	LATTA.002A	7335

20995 7590 06/19/2006

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EXAMINER	
BELYAVSKYI, MICHAIL A	
ART UNIT	**PAPER NUMBER**
1644	

DATE MAILED: 06/19/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Notification of Non-Compliant Appeal Brief (37 CFR 41.37)	Application No. 10/660,924	Applicant(s) LATTA, PAUL P.	
	Examiner Michail A. Belyavskyi	Art Unit 1644	

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

The Appeal Brief filed on *11/04/05* is defective for failure to comply with one or more provisions of 37 CFR 41.37.

To avoid dismissal of the appeal, applicant must file anamended brief or other appropriate correction (see MPEP 1205.03) within **ONE MONTH or THIRTY DAYS** from the mailing date of this Notification, whichever is longer. **EXTENSIONS OF THIS TIME PERIOD MAY BE GRANTED UNDER 37 CFR 1.136.**

1. ☐ The brief does not contain the items required under 37 CFR 41.37(c), or the items are not under the proper heading or in the proper order.

2. ☐ The brief does not contain a statement of the status of all claims, (e.g., rejected, allowed, withdrawn, objected to, canceled), or does not identify the appealed claims (37 CFR 41.37(c)(1)(iii)).

3. ☐ At least one amendment has been filed subsequent to the final rejection, and the brief does not contain a statement of the status of each such amendment (37 CFR 41.37(c)(1)(iv)).

4. ☒ (a) The brief does not contain a concise explanation of the subject matter defined in each of the independent claims involved in the appeal, referring to the specification by page and line number and to the drawings, if any, by reference characters; and/or (b) the brief fails to: (1) identify, for each independent claim involved in the appeal and for each dependent claim argued separately, every means plus function and step plus function under 35 U.S.C. 112, sixth paragraph, and/or (2) set forth the structure, material, or acts described in the specification as corresponding to each claimed function with reference to the specification by page and line number, and to the drawings, if any, by reference characters (37 CFR 41.37(c)(1)(v)).

5. ☐ The brief does not contain a concise statement of each ground of rejection presented for review (37 CFR 41.37(c)(1)(vi))

6. ☐ The brief does not present an argument under a separate heading for each ground of rejection on appeal (37 CFR 41.37(c)(1)(vii)).

7. ☐ The brief does not contain a correct copy of the appealed claims as an appendix thereto (37 CFR 41.37(c)(1)(viii)).

8. ☐ The brief does not contain copies of the evidence submitted under 37 CFR 1.130, 1.131, or 1.132 or of any other evidence entered by the examiner **and relied upon by appellant in the appeal**, along with a statement setting forth where in the record that evidence was entered by the examiner, as an appendix thereto (37 CFR 41.37(c)(1)(ix)).

9. ☐ The brief does not contain copies of the decisions rendered by a court or the Board in the proceeding identified in the Related Appeals and Interferences section of the brief as an appendix thereto (37 CFR 41.37(c)(1)(x)).

10. ☐ Other (including any explanation in support of the above items):

———

Christina Chan

CHRISTINA CHAN
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

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SUPPLEMENTAL APPEAL BRIEF

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Sir:

In accordance with the Notice of Appeal filed August 17, 2005 and in response to the Notification of Non-Compliant Appeal Brief mailed June 10, 2006, Applicant submits this Supplemental Appeal Brief.

TABLE OF CONTENTS

I. REAL PARTY IN INTEREST

Pursuant to 37 C.F.R. §1.192, Appelants hereby notify the Board of Patent Appeals and Interferences that the real party in interest is the inventor for this application, Paul P. Latta, 33 Santa Cruz Aisle, Irvine, CA 92606.

II. RELATED APPEALS AND INTERFERENCES

Appellants are unaware of any related appeals or interferences.

III. STATUS OF CLAIMS

The above-identified application was filed with 1 claim. In a Preliminary Amendment, filed April 1, 2004, Claim 1 was canceled and new claims 2-9 were introduced. Claims 2-9 were finally rejected by the Examiner in a Final Office Action mailed May 18, 2005. Accordingly, Claims 2-9 are the subject of this appeal. The Claims are attached hereto as Appendix A.

IV. STATUS OF AMENDMENTS

Appellants filed a Pre-Appeal Brief Request for Review on August 17, 2005. No amendments have been filed subsequent to the final rejection.

V. CONCISE EXPLANATION OF SUBJECT MATTER OF INDEPENDENT CLAIM

Claim 2 is the only independent claim in this appeal. The subject matter of this claim relates to Appellant's discovery of a method of preventing onset of Type I diabetes in a mammal predisposed to Type I diabetes. See Applicant's specification at page 10, line 12-21 and page 19, lines 1-19. The method includes implanting a dose of insulin-producing cells into an implantation site in said mammal. See page 4, lines 3-4. The cells that are implanted are encapsulated in a biologically-compatible membrane. See page 3, lines 30-31 and page 8, lines 2-4. The cells are implanted prior to onset of Type I diabetes. See page 10, lines 15-16. The dose of implanted cells is at least one order of magnitude less than that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes. See page 4, lines 26-27; page 9, lines 9-11, page 12, lines 26-29 and page 19, lines 13-19.

When using the method of this claim, the encapsulated cells shed antigens through the capsule membrane into the blood stream of a host, while being protected from the attack by the host immune system. Over time, the host immune system gets tolerized to the implanted cells and diverts its response away from the process of destruction of self islet cells. See, specification, page 8, lines 2-29; page 10, lines 12-21; and page 17, lines 3-11.

Each limitation of Claim 2, the only independent claim, is supported by the Specification as filed as follows:

LIMITATION	PAGE:LINES
A method of preventing onset of Type I diabetes in a mammal predisposed to Type I diabetes, comprising	10:12-21; 19:1-19
implanting a dose of insulin-producing cells	4:3-4
encapsulated in a biologically-compatible membrane into an implantation site in said mammal	3:30-31; 8:2-4
prior to onset of Type I diabetes,	10:15-16
wherein said dose is at least one order of magnitude less than that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes.	4:26-27; 9:9-11; 12:26-29; 19:13-19

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. The Examiner has rejected Claims 2-9 under 35 U.S.C. §112, first paragraph for lack of enablement[1]. In particular, the Examiner has maintained that the Specification and the Declarations by Dr. Scharp showing efficacy of the claimed method in preventing type I diabetes in a mouse model of diabetes (non-obese diabetic, NOD mice) are not predictive of the outcome of using the claimed method for the prevention of Type I diabetes in human[2].

[1] Final OA, mailed 05/18/05, page 3, paragraph 5.
[2] *Id.*

2. The Examiner has also rejected Claims 2-9 under 35 U.S.C. §112, first paragraph as having New Matter[3].

VII. ARGUMENT

1. The Examiner has maintained rejections of Claims 2-9 under 35 USC §112, first paragraph, as being non-enabling for a method of preventing onset of Type I diabetes in a mammal. The reasons the Examiner has maintained for the rejection are: **A)** unpredictability of the prevention of diabetes in human from the murine data; **B)** need for screening for susceptible individuals; **C)** need for undue experimentation to determine screening and testing protocols to demonstrate the efficacy of the claimed invention[4,5]. Each of these reasons is addressed below:

a. Prevention of diabetes in human is reasonably predictable from *in vivo* data obtained in NOD mice.

The first reason the Examiner stated for the rejection is unpredictability of the prevention of diabetes in a human from the murine data. The Examiner recognized that "[s]ubstantiating evidence may be in the form of animal tests, which constitute recognized screening procedures with clear relevance to efficacy in humans", citing *Ex parte Krepelka*, 231 U.S.P.Q. 746 and *Ex parte Maas*, 9 U.S.P.Q.2d 1746. Nevertheless, the Examiner has insisted that it is unpredictable from the *in vivo* murine data whether the method of the invention can be used in mammals including humans[6]. Therefore, the Examiner is applying a much stricter standard than required by law.

According to MPEP 2107.03:

> "Evidence does not have to be in the form of data from an art-recognized animal model for the particular disease or disease condition to which the asserted utility relates. Data from any test that the applicant reasonably correlates to the asserted utility should be evaluated substantively. Thus, an applicant may provide data generated using a particular animal model with an appropriate explanation as to why that data supports the asserted utility. The absence of a certification that the test in question is an industry-accepted model is not dispositive of whether

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[3] *Id.*, at page 6, paragraph 7.
[4] Office Action, mailed 12/16/04, page 3, paragraph 7.
[5] Final OA, mailed 05/18/05, page, paragraph 5.
[6] Final OA, mailed 05/18/05, page, paragraph 5.

data from an animal model is in fact relevant to the asserted utility. Thus, if one skilled in the art would accept the animal tests as being reasonably predictive of utility in humans, evidence from those tests should be considered sufficient to support the credibility of the asserted utility."

"Office personnel should be careful not to find evidence unpersuasive simply because no animal model for the human disease condition had been established prior to the filing of the application. See In re Chilowsky, 229 F.2d 457, 461, 108 USPQ 321, 325 (CCPA 1956) ("The mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it."); In re Wooddy, 331 F.2d 636, 639, 141 USPQ 518, 520 (CCPA 1964) ("It appears that no one on earth is certain as of the present whether the process claimed will operate in the manner claimed. Yet absolute certainty is not required by the law. The mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it.")".

In these cases, it is important to note that the Food and Drug Administration has promulgated regulations that enable a party to conduct clinical trials for drugs used to treat life threatening and severely-debilitating illnesses, even where no alternative therapy exists. See 21 CFR 312.80-88 (1994). Implicit in these regulations is the recognition that experts qualified to evaluate the effectiveness of therapeutics can and often do find a sufficient basis to conduct clinical trials of drugs for incurable or previously untreatable illnesses. Thus, affidavit evidence from experts in the art indicating that there is a reasonable expectation of success, supported by sound reasoning, usually should be sufficient to establish that such a utility is credible.

As discussed in the Response to Office Action mailed December 16, 2004, in connection with compliance with the written description requirement, the specification describes every feature of the claimed invention[7]. One skilled in the art would have no difficulty carrying out the steps for making and using the invention based on this description. To establish that one actually carrying out these steps could successfully achieve the claimed result, i.e. prevention of diabetes, Applicant submitted on April 1, 2004 a first Declaration of Dr. David Scharp[8] and on April 29,

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[7] Amendment and Response to Office Action of December 16, 2004, filed April 29, 2005, page 4, paragraph 3.
[8] Declaration of David Scharp, dated November 25, 2003 and filed April 11, 2004.

2005 a second Declaration of Dr. David Scharp[9] showing that NOD mice receiving the treatment described in the specification were indeed prevented from becoming diabetic.

Moreover, these Declarations and the references submitted by Applicant on April 29, 2005[10] establish the validity of the NOD mouse model in connection with human diabetes. The provided support in the form of affidavit from the expert in the art (Second Declaration, CV) as well as scientific publications (Appendices 1 and 2) indicates that the NOD mice is at present the standard model for studying Type I diabetes prevention and treatment.

The NIH recognize the NOD mouse as the model animal for diabetes and maintains a research colony and data base on these animals for researchers. The NIH state "The NOD mouse, which spontaneously develops type 1 diabetes, is a valuable animal model that is used extensively in research exploring the etiology, prevention, and treatment of this disease. It is a vital research tool for testing promising prevention and treatment strategies at the preclinical level."[11].

Furthermore, Hanninen et al. states: "The non-obese diabetic (NOD) mouse is the most widely used animal model of T1DM. [...] research in non-obese diabetic mice has led to the discovery of new strategies of diabetes prevention that are now in human clinical trials".[12] The authors further presented a whole list of current clinical trials based on strategies developed in NOD mice[13].

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[9] Second Declaration of David Scharp, dated April 28, 2005 and filed April 29, 2005.
[10] Appendices 1: http://www.niaid.nih.gov/dait/NODmice.htm; and 2: Hanninen et al. 2003 "Development of new strategies to prevent type I diabetes: the role of animal models" *Annals of Medicine* **35**:546-563, submitted with the Second Declaration of David Scharp, dated April 28, 2005.
[11] http://www.niaid.nih.gov/dait/NODmice.htm.
[12] Hanninen et al. 2003 "Development of new strategies to prevent type I diabetes: the role of animal models" *Annals of Medicine* **35**:546-563: page 546, right column, last paragraph through page 547, left column.
[13] *Id.*, Table 2.

While it is true that questions have been raised whether the NOD model is absolutely predictive of treatment of humans[14], such an absolute correlation with human disease is not required to support enablement. MPEP 2107.03 further provides:

> The applicant does not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty, nor does he or she have to provide actual evidence of success in treating humans where such a utility is asserted. Instead, as the courts have repeatedly held, all that is required is a reasonable correlation between the activity and the asserted use.

Therefore, using the proper standard set forth in the MPEP, the evidence provided by Applicant clearly supports that one skilled in the art would accept the NOD model as reasonably correlating to the condition in human.

b. Screening for susceptible individuals is well-known and routine

The Examiner also objected to the enablement provided by the specification based on an alleged failure to disclose how to screen for susceptible individuals. However, the Specification as filed[15], provides information that "in diabetes, the use of immune marker autoantibodies to establish preclinical diabetes has been well studied". The Specification cites Palmer, 1993 *Diabetes Rev.* **1**(1):104-116 in support of this statement. In addition, Applicant provided several references to support the position that screening for individuals susceptible to developing type I diabetes has been well-established in the art at the time the invention was made: Bonifacio et al. 1995 "Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity", *Diabetologia* **38**:816-22; Lee et al. 1995 "Relationships among 64k autoantibodies, pancreatic beta-cell function, HLA-DR antigens and HLA-DQ genes in patients with insulin-dependent diabetes mellitus in Korea", *Korean J. Intern. Med.* **10**:1-9; Bingley et al. 1994 "Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives", *Diabetes* **43**:1304-10; Zimmet et al. 1994 "Autoantibodies to glutamic acid decarboxylase and insulin in islet cell antibody positive presymptomatic type 1 diabetes mellitus: frequency and segregation by age and gender", *Diabet Med.* **11**:866-71; Christie et al. 1994 "Antibodies to islet

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[14] Final Office Action, mailed May 18, 2005, page 3, paragraph 5.

37k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity", *Diabetes* **43**:1254-9; Tuomilehto et al. 1994 "Antibodies to glutamic acid decarboxylase as predictors of insulin-dependent diabetes mellitus before clinical onset of disease", *Lancet* **343**:1383-5; Zimmet et al. 1994 "Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency", *Diabet Med.* **11**:299-303[16].

"The specification need not disclose what is well known in the art." *See, e.g., In re Buchner,* 18 USPQ2d 1331 (Fed. Cir. 1991). Thus, there was no requirement for Applicants' specification to contain a thorough description of the well known techniques that were well-established as of the effective filing date. Accordingly, one skilled in the art would have no difficulty identifying suitable subjects for the treatment of the present invention.

c. Experimentation to determine screening and testing protocols to demonstrate the efficacy of the claimed invention in not undue

The Examiner also maintained that undue experimentation would be required to determine screening and testing protocols. However, as established in the Second Declaration of David Scharp, M.D.[17], methods for determining whether normoglycemia is present have been exceedingly well known for many years; thus, only routine blood glucose monitoring would be required to demonstrate the efficacy of the claimed invention. Furthermore, the Applicants provided a sufficient showing in the Response to the Office Action mailed December 16, 2004, that screening for individuals susceptible to Type I diabetes is well-known and routine, amount of insulin-producing cells needed to be implanted to achieve normoglycemia is also well-known, as are the tests to determine whether normoglycemia is present[18].

MPEP 2164.01(c) establishes that in order to meet the enablement requirement, one skilled in the art need only be able to discern an appropriate dosage or method of use without undue experimentation based on knowledge of compounds having similar physiological or

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[15] Specification, page 19, lines 9-12.
[16] All references submitted with the Amendment and Response to Office Action of December 16, 2004.
[17] submitted on April 29, 2005, see page 2.

biological activity. Here, the Specification at page 12, lines 26-30 clearly indicates that a dose of implanted insulin-producing cells to induce tolerance is one or two orders of magnitude less than a full dose of implant which provides adequate insulin production for normoglycemia. The full dose for achieving normoglycemia has been well worked out for many years. Accordingly, one skilled in the art would have no difficulty in obtaining the correct dose for any given individual. Accordingly, no undue experimentation would be required to practice the claimed invention. Therefore, the rejection of Claims 2-9 as non-enabled is clearly improper.

2. The Examiner has improperly rejected Claims 2-9 for allegedly containing New Matter. Specifically, The Examiner has stated that the limitation added to Claim 2: "wherein said dose is at least one order of magnitude less than that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes" is not supported by the passages of the Specification pointed to by the Applicant[19].

The Specification states at page 19, lines 13-19 that the dose for prevention is the same as the dose for tolerization that is discussed earlier in the specification in connection with disease treatment. At page 4, lines 26-27, the specification clearly states that "the tolerizing dose is one or two orders of magnitude less than the curative dose", while at page 9, line 9-11, the specification also clearly states that "[a]s for the bolus tolerizing dose, the incremental tolerizing dose is typically one or two orders of magnitude lower than the curative dose. Thus, the specification clearly establishes that the dose for prevention of diabetes is "at least one order of magnitude less than that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes." Therefore, there is clear support in the Specification as filed for Claim 2, and its rejection over "New Matter" is improper.

Conclusion

In view of the arguments presented above, Appellants submit that the Specification as filed enables a person with an ordinary skill in the art on how to make and use the invention.

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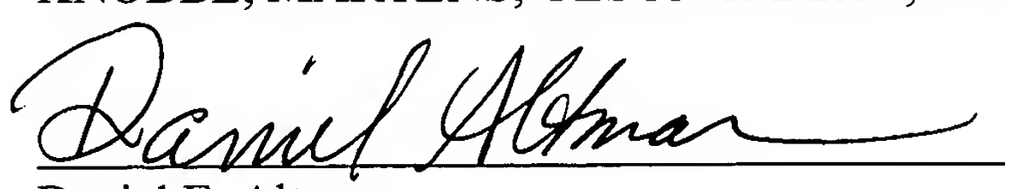
[18] Amendment and Response, filed April 29, 2005, pages 7-8.
[19] Final Office Action, mailed May 18, 2005, paragraph 7.

Appellants further submit that Claims 2-9 are fully supported by the Specification as filed and do not constitute New Matter.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP.



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VIII. CLAIMS APPENDIX

1. **(Canceled)**

2. **(Previously presented)** A method of preventing onset of Type I diabetes in a mammal predisposed to Type I diabetes, comprising implanting a dose of insulin-producing cells encapsulated in a biologically-compatible membrane into an implantation site in said mammal prior to onset of Type I diabetes, wherein said dose is at least one order of magnitude less than that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes.

3. **(Previously presented)** The method of Claim 2, wherein said cells are from a primary cell source.

4. **(Previously presented)** The method of Claim 3, wherein said cells are pancreatic islet cells.

5. **(Previously presented)** The method of Claim 2, wherein said cells are encapsulated in a conformal coating.

6. **(Previously presented)** The method of Claim 5, wherein said conformal coating comprises polyethylene glycol (PEG).

7. **(Previously presented)** The method of Claim 2, wherein the insulin-producing cells are from the same species as the mammal.

8. **(Previously presented)** The method of Claim 2, wherein Type I diabetes is prevented without continuous immunosuppression.

9. **(Previously presented)** The method of Claim 2, wherein the cells are implanted intraperitoneally.

IX. EVIDENCE APPENDIX

1. Specification as filed;

2. Declaration of David Scharp, signed November 25, 2003 and filed April 1, 2004 with Appendix A;

3. Office Action, mailed December 16, 2004;

4. Response to Office Action, filed April 29, 2005;

5. Second Declaration of David Scharp, signed April 28, 2005 and filed April 29, 2005 (with Appendix 1 and Appendix 2), acknowledged by the Examiner in the Final Office Action mailed May 18, 2005;

6. Appendix 1 - http://www.niaid.nih.gov/dait/NODmice.htm printout;

7. Appendix 2 - Hanninen et al. 2003 "Development of new strategies to prevent type I diabetes: the role of animal models" *Annals of Medicine* **35**:546-563;

8. Bonifacio et al. 1995 "Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity", *Diabetologia* **38**:816-22, filed April 29, 2005 and entered in the record by the Examiner on May 11, 2005 in the Final Office Action mailed May 18, 2005;

9. Lee et al. 1995 "Relationships among 64k autoantibodies, pancreatic beta-cell function, HLA-DR antigens and HLA-DQ genes in patients with insulin-dependent diabetes mellitus in Korea", *Korean J. Intern. Med.* **10**:1-9, abstract only filed April 29, 2005, filed April 29, 2005 and entered in the record by the Examiner on May 11, 2005 in the Final Office Action mailed May 18, 2005;

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12. Christie et al. 1994 "Antibodies to islet 37k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity", *Diabetes* **43**:1254-9, filed April 29, 2005, filed April 29, 2005 and entered in the record by the Examiner on May 11, 2005 in the Final Office Action mailed May 18, 2005;

13. Tuomilehto et al. 1994 "Antibodies to glutamic acid decarboxylase as predictors of insulin-dependent diabetes mellitus before clinical onset of disease", *Lancet* **343**:1383-5, filed April 29, 2005, filed April 29, 2005 and entered in the record by the Examiner on May 11, 2005 in the Final Office Action mailed May 18, 2005;

14. Zimmet et al. 1994 "Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency", *Diabet Med.* **11**:299-303, filed April 29, 2005, filed April 29, 2005 and entered in the record by the Examiner on May 11, 2005 in the Final Office Action mailed May 18, 2005;

15. Final Office Action, mailed May 18, 2005 with 1 reference cited (Atkinson M.A. et al. 1999 "The NOD mouse model of type 1 diabetes: As good as it gets?" Nature Med. 5:601-604).

X. RELATED PROCEEDINGS APPENDIX

There are no decisions rendered by a court or the Board in any related proceedings identified above.

INDUCTION OF IMMUNOLOGICAL TOLERANCE

Related Applications

This application is a continuation of Application Serial No. 09/226,742 filed January 7, 1999 which is a continuation of Application Serial No. 09/049,757 filed March 27, 1998 now abandoned, which was a continuation of Application Serial No. 08/736,413 filed on October 24, 1996 now abandoned, which claims the benefit of priority under 35 U.S.C. 119(e) of Provisional Application No. 60/005,877 filed October 26, 1995.

Field of the Invention

The present invention relates to the induction of immunological tolerance to foreign cells, tissues and organs. More specifically, the invention relates to implantation of a tolerizing dose of cells or tissues encapsulated in a membrane in a mammal to establish immunological tolerance thereto.

Background of the Invention

For some human diseases, including heart and liver failure, organ transplantation is the only alternative to certain death. While there were only 4,843 organ donors in the U.S. in 1993, there were 2,866 heart and 3,040 liver failure patients on the waiting list for these organs (*UNOS Update*, **10**(2), 1994). Thus, because of timing and tissue matching problems, many patients die each year for lack of an available organ. For those lucky enough to receive an organ, the results are still less than ideal. The transplant procedure constitutes major surgery which is associated with attendant risks and is exceedingly expensive. After the surgery, the patient must be placed on a regimen of immunosuppressive drugs to keep the immune system from destroying the transplanted organ. As a consequence, the patient's entire immune system is suppressed for the rest of his life, significantly lowering his defenses against other serious disease threats such as infections, viruses or cancers.

For other diseases including kidney failure, pancreas failure and cystic fibrosis, transplantation has a lower mortality and morbidity rate than any alternative therapy. Even with its attendant problems of organ scarcity, surgical risk, high cost and permanent immunosuppression, for some of these cases it is still a more practical therapy than any alternative. The physician's choice in these cases is dependent on many variables including age, general health, severity of the condition, availability of organs and others factors. In 1994, there were 25,033 patients on the waiting list for human kidneys, 181 for pancreases and 1,250 for lungs (*UNOS Update*, **10**:2, 1994).

For still other diseases, transplantation is known to be effective, although its attendant problems preclude its practical therapeutic use. This is true for many of the kidney, pancreas and lung patients described above. It is also true where whole pancreas transplantation can cure diabetes or liver transplantation can cure hemophilia but the risks outweigh the rewards.

Recently, for certain disease states; tissue transplants, as opposed to whole organ transplants, have been shown to be therapeutic in animals and even in man (Scharp et al., *Transplantation*, 51:76-85, 1991). Tissue transplantation requires full immunosuppression and carries the same risks and problems as already discussed for whole organ immunosuppression. The following treatments address the rejection of the transplanted tissue.

One implantation method involves pre-inoculation in the thymus with a small dose of cells, full temporary immunosuppression, then a full therapeutic dose at another site (Posselt et al., *Annals of Surgery*, 214:363-373, 1991). First, this has only been shown to work in rodents to date. No large animal or human test has been successful. Second, the human adult thymus is shrunken and may not be practical to treat with an adequate pre-dose. Third, the immunosuppression step, while temporary, does subject the patient to risks for that period of time. Fourth, it is not known whether a fully therapeutic dose will be tolerated, (i.e. not rejected) in humans. Fifth, this procedure may not protect against autoimmune destruction even if it does prevent rejection.

Another method of preventing rejection is irradiation of the recipient's bone marrow immune cells, implantation of bone marrow cells from the donor, then implantation of a full therapeutic dose of tissue or organ from the same donor (Illstad et al., *J. Exp. Med.*, 174:467-478, 1991). First, this has not been shown to work for tissue transplants in humans. Second, irradiation of immune cells, either partial or whole body, carries serious risks. Third, it is not known if the immune system will adequately protect from other threats. Fourth, it is not known if the method will protect from both rejection and autoimmune destruction in those disease states.

A further method of treatment to prevent rejection is by using monoclonal antibodies to suppress certain parts of the immune system (Andersson et al., *J.*

Autoimmun., 4:733-742, 1991). These tests have only been performed in rodents so it is not known if they would succeed in humans. Also, it is not known if the proper monoclonal antibody could be identified and created for each different disease state. In addition, the overall affect of these agents on the human immune system is not known.

Still another method of preventing rejection is encapsulation of the transplanted tissue in a semi-permeable membrane device which allows oxygen, nutrients and other small molecules to pass but prevents entry of large immune system cells (Lacy et al., *Science*, 254:1782-1784, 1991; Sullivan et al., *Science*, 252:718-721, 1991). There are several unresolved problems associated with this method. First, none of these devices has been shown to protect a therapeutic transplant in humans. To be suitable for human use, the material must be biocompatible; it must be sufficiently strong to last a long time when implanted; its porosity must be exactly correct to allow survival and function of the enclosed cells while keeping out cells and perhaps antibodies of the immune system; and finally, the device itself must be large enough to contain enough cells for a fully therapeutic implant and yet small enough to allow for some reasonable method of implantation which causes no damage to other internal organs.

To date, there has been very little effort to use transplantation as a potential prevention of disease due to all of the problems associated with transplantation as previously described. In addition, it is not yet known where transplantation can actually prevent a disease from occurring other than the obvious case of whole organ failures. Moreover, for many disease states, it is not known who will be afflicted. There is some evidence that interventional transplantation can have some preventive effect in rodents (Miller et al., *J. Neurol. Immunol.*, 46:73-82, 1993; van Vollenhoven et al., *Cell. Immunol.*, 115:146-155, 1988). Thus, a major role for preventive transplantation has not been investigated.

Summary of the Invention

One embodiment of the invention is a method of creating immunological tolerance to foreign cells, tissues or organs in a mammal, comprising the step of implanting in the mammal a tolerizing dose of foreign cells or tissue encapsulated in a biologically compatible permselective membrane. The method may additionally

comprise the step of administering to the mammal a curative dose of corresponding unencapsulated cells, tissue or organ. Advantageously, the mammal is a human, canine or feline. Preferably, the tolerizing cells are insulin-secreting cells; more preferably, they are pancreatic islet cells. According to one aspect of this embodiment, the membrane comprises polyethylene glycol. Preferably, the curative dose is between one and two orders of magnitude greater than the tolerizing dose. Advantageously, the tolerizing and curative doses are from the same species as the mammal. Alternatively, the tolerizing and curative doses are from a species different from the mammal. Preferably, the tolerizing and curative doses are porcine. The method may further comprise the step of administering one or more anti-inflammatory agents to the mammal prior to, at the same time as, or subsequent to administration of the curative dose. Preferably, the membrane has a molecular weight cutoff of about 150 kDa or less. Alternatively, the membrane has a pore size of about 0.4 μm or less. The membrane may also have a pore size of about 0.2 μm or less. Advantageously, when the tolerizing and curative doses are from a different species, the membrane has a molecular weight cutoff of about 150 kDa or less. Preferably, the tolerizing step is subcapsular, subcutaneous, intraperitoneal or intraportal and the curative step is intraperitoneal, intraportal or subcutaneous. The tolerizing dose may also be administered incrementally.

The present invention also provides a method of treating diabetes in a mammal in need thereof, comprising the steps of:

implanting in the mammal a tolerizing dose of foreign insulin-secreting cells encapsulated in a biologically compatible permselective membrane; then

administering to the mammal a curative dose of corresponding unencapsulated insulin-secreting cells.

Preferably, the mammal is a human, canine or feline. Advantageously, the tolerizing dose is one to two orders of magnitude less than the curative dose. In another aspect of this preferred embodiment, the membrane comprises polyethylene glycol. Advantageously, the insulin-secreting cells are pancreatic islet cells. Preferably, the mammal and the insulin-secreting cells are from the same species. Alternatively, the mammal and the insulin-secreting cells are from different species. Preferably, the

tolerizing and curative doses are porcine. The method may further comprise the step of administering one or more anti-inflammatory agents to the mammal prior to, at the same time as, or subsequent to administration of the curative dose. Advantageously, the membrane has a molecular weight cutoff of about 150 kDa or less. Alternatively, the membrane has a pore size of less than about 0.4 μm.

Brief Description of the Drawings

Figure 1 is plane view illustrating the key properties of the membrane enclosing the cells. The membrane may be configured into many different device designs.

Figure 2 is a plane view of one design of the invention, wherein two layers of the membrane are used in a flat sheet configuration where cells are "sandwiched" in between the two membranes and then the ends are sealed.

Figure 3 is a tubular view of one design of the invention, wherein the membrane is cast or rolled into a tubular configuration. The cells are loaded in the lumen and the ends are sealed.

Figure 4 is a spherical view of one design of the invention, wherein the membrane is cast in a spherical configuration and cells may be encased one in each device (microcapsule) or many in a device (macrocapsule).

Figure 5 is a graph showing blood glucose levels in mice implanted with a tolerizing dose of 100 encapsulated NIT insulinoma aggregates.

Figure 6 is a graph showing blood glucose levels in mice implanted with a tolerizing dose of 50 encapsulated NIT insulinoma aggregates.

Figure 7 is a graph showing blood glucose levels in non-tolerized control mice.

Detailed Description of the Preferred Embodiments

Goals of the Invention

The problems discussed in the foregoing Background of the Invention have previously not been solved for either micro or macroencapsulation of cells in humans. The present invention overcomes these problems associated with transplantation. Thus, one goal of the invention is to eliminate the critical problems of transplantation in cases where whole organ transplantation is the only alternative to certain death. These are cases of heart or liver failure. The major advantage of the invention process

for this application is that it eliminates the shortage of organs for the patients by making animal organs acceptable in humans. While there are only about 4,800 human organ donors in the U.S. each year, the supply of animal organs for transplant is not limited. The reason that animal organs are not presently used is that they are acutely rejected when transplanted into humans even with immunosuppression. Second, continuous immunosuppression is not required in the process of the invention, thus eliminating the risk of exposing the patient to other serious diseases while the immune system is suppressed. Third, the cost of organ transplantation is drastically reduced because of the unlimited supply of organs and because the continuous use of immunosuppressive drugs is not required.

A second goal of the invention is to make organ transplantation a safe, effective, practical therapy for those cases of disease where it is known now to be therapeutic but the risks associated with it prevent its widespread therapeutic use. Examples of these disease cases are kidney failure, pancreas failure and cystic fibrosis (lung failure). In these cases the advantages of the process of the invention eliminate the major obstacles. First, by making animal organs tolerated in humans the shortage of organs for these transplant needs is solved. Second, by eliminating the need for continuous immunosuppression, these patients are not exposed to other serious disease threats without a fully functioning immune system. Third, because of plentiful organs and no continuous immunosuppression, the cost of this transplant procedure would be greatly reduced.

A third goal of the invention is to make cell or tissue transplants, as opposed to whole organ transplants, a practical therapy in cases where cells or tissue alone can cure a disease state by providing a lacking or deficient protein, enzyme or peptide. Examples of these cases are insulin-secreting islet cells for Type I diabetes, Factor VIII-secreting hepatic cells for hemophilia, dopamine-secreting adrenal chromaffin cells for Parkinson's disease and collagen for arthritis. A significant advantage of the process of the invention for these cases is that animal tissue or genetically engineered tissue expressing an absent or deficient protein of interest can be used if human tissue is scarce. In addition, cell types other than the normal protein-secreting cells can be engineered to secrete the protein of interest. For example, myoblasts can be

engineered by standard methods to secrete insulin. The use of such cells is also within the scope of the present invention. Continuous immunosuppression is not needed to protect the transplanted tissue and the costs would be reduced. Thus, even if pre-inoculation into the thymus with immunosuppression or irradiation of bone marrow with immunosuppression or monoclonal antibodies could be identified and produced for many disease states or encapsulation of fully therapeutic doses of tissue in some membrane device can overcome many technical problems, the process of the invention is a safer and more practical therapy than any of these.

A fourth goal of the invention is the treatment of autoimmune diseases including diabetes, Alzheimer's, arthritis, multiple sclerosis, myasthenia gravis and systemic lupus erythematosus. In these diseases, the body's immune system attacks and destroys one's own tissue. By using the process of the invention, the immune system can be induced to accept grafted tissue or organs to replace those that have been destroyed without the autoimmune destruction of the newly transplanted graft. The advantage of this process is that organ rejection and autoimmune destruction are two completely different phenomena so that even with systems that may prevent rejection, in autoimmune diseases the grafts may still be destroyed by a different means. The process of the invention addresses both problems.

A fifth goal of the invention is to make transplantation a practical therapy to prevent certain diseases from ever occurring, as well as treating existing diseases as previously discussed. The advantage of the process that makes this possible is the immunomodulation effect which stops or prevents the immune system from destruction of self tissue. Thus, for all autoimmune disorders, the process can be used to intervene in the course of the disease at a critical point before the immune system is initiated into self-destruction of tissue that is necessary for normal body function.

As will be apparent from the ensuing detailed description of the invention, the present invention meets all of these goals. Additionally, the present invention also provides a number of advantages which would not have been readily apparent to one having ordinary skill in the art.

Overview

The present invention is a two step process. In the first step, a small number of cells or tissue is implanted into a mammal inside a device made of a biocompatible "permselective" membrane which protects the implanted cells from the mammal's immune system while at the same time allowing the cells to survive. A permselective membrane is one having a pore size selected so that it is small enough to prevent the entry of immunological factors such as cells or antibodies, yet large enough to allow the free passage of oxygen, nutrients and other molecules needed to sustain the transplanted cells. In addition, the membrane pores must allow the passage of antigens which are shed from the transplanted cells and prevent the entry of large immune system cells and antibodies. In a preferred embodiment, the mammal is a human. Alternatively, the mammal is a canine or feline.

One of ordinary skill in the art can readily determine the proper pore size for the permselective membrane for any particular application of the present invention. It is preferable to use the largest pore size possible to prevent the entry of the undesirable elements because the larger pores allow better diffusion of the desirable elements such as nutrients and oxygen across the membrane. Smaller pore sizes (e.g. those excluding molecules greater than 100,000 daltons) are not necessarily a problem for diffusion as has been shown in long-term survival of cells in a 50,000 dalton membrane *in vivo* implant (Lacy et al., *Science* **254**:1782-1784, 1991).

Antigens shed from the transplanted cells pass through the permselective membrane into the body of the recipient where they are fully exposed to the immune system. The immune system will recognize these antigens as "foreign" and destroy them. This process will continue for some time with the immune system constantly destroying the shed antigens but not able to destroy the source which is the cells protected in the encapsulation device. In time, the immune system will begin to become tolerant of these antigens because they do no actual damage in the body and the constant source cannot be destroyed. At this time, the immune system is tolerant to that particular cell type from that particular donor.

Next, the second stage of the process is enacted. Now a fully therapeutic (curative) dose of cells, tissue or whole organ from the same donor as the tolerizing

dose is implanted in the recipient for cure of the disease. Since this implant, whether cells, tissue or organ, is from the same donor as the small dose, it is recognized by the immune system as "self" and a rejection response is not elicited. The immune system is fully tolerant to the new implant. In one embodiment, the tolerizing dose is given as a single (bolus) dose. Alternatively, the tolerizing dose may be administered incrementally over several weeks or months. In a preferred embodiment, the incremental tolerizing dose is the same as the bolus dose, only spread out in even increments. In another embodiment, the total incremental tolerizing dose is one to three times the bolus tolerizing dose. As for the bolus tolerizing dose, the incremental tolerizing dose is typically one to two orders of magnitude lower than the curative dose.

In addition to eliminating continuous immunosuppression, this process makes animal organs and cells available for human implants (xenografts). Presently, these organs or tissues are acutely rejected in humans because of the wide immunological barriers between the species. With the process of the invention, even animal tissue will be tolerated because tolerance is induced gradually over time. The availability of animal organs for human use will save many thousands of lives each year which are now lost due to the shortage of available human organs for transplantation. In addition, this process will allow transplant therapy for autoimmune diseases such as diabetes, arthritis, myasthenia gravis and multiple sclerosis. This is possible because as the immune system is tolerized to the new tissue by the initial small implant, the self-destructive autoimmune process is suppressed. So, for diseases requiring organs or cellular transplants, this process eliminates current shortages by making unlimited supplies of animal organs and cells available, eliminates the need for continuous immunosuppression, and protects the transplants from both rejection and autoimmune destruction. One particularly preferred source of xenograft cells or tissue for both the tolerization and curative steps is porcine cells or tissue.

Even with the tolerizing effect of the xenograft, because of the wide species differences, an initial inflammatory reaction may occur in response to the curative dose. Thus, in one embodiment of the invention, the xenograft recipient is administered one or more anti-inflammatory agents. The anti-inflammatory agent is

administered either systemically or locally at the implantation site. The agent may be administered prior to the implant, at the time of implantation or subsequent to the implant for a time necessary to overcome the initial inflammatory reaction. The agents may be over-the-counter preparations such as acetaminophen or ibuprofen, or a specific immunosuppressive agent such as Cyclosporine (Sandoz) or Imuran (azathioprine, Burroughs-Wellcome). The agent may also block the binding of a particular antigen such as CTLA4Ig (Bristol Myers Squibb). The amount of anti-inflammatory agent to be administered is typically between about 1 mg/kg and about 10 mg/kg. The extent of inflammation will determine whether the administration of such an agent(s) is necessary. The need for such agents is only temporary and not required for the ongoing survival and function of the transplant.

The process of the invention can also be used to prevent certain diseases, particularly autoimmune disorders. In these cases the process is as follows. First, patients at high risk for the disease or already in the very early phase of the disease are identified. At the critical time of the onset, the process is intervened with the small encapsulated tissue. For example, islets are used for Type I diabetes and collagen is used for arthritis. This implant of foreign tissue immediately diverts the attention of the immune system to the new foreign invader and it begins the process to destroy this new threat. Because of this diversion, the process of self-destruction of desirable tissue that was just beginning is suppressed, then abandoned, then forgotten. It is, in essence, "switched off" and the damage is prevented.

Implantation of cells to treat existing diseases

The first step of this method involves acquiring small amounts of cellular tissue for the initial tolerizing implant. The method in which tissue is obtained depends on the type of tissue needed, the source of the tissue, the donor, and the amount of tissue needed. These methods are generally well known by those skilled in the art of tissue digestion, separation, purification, culture, and the like. The following examples are only used to illustrate that these methods are readily available.

Islet cells for treatment of diabetes

Islets are small clusters of cells located in the pancreas of mammals. They are composed of alpha cells which make and secrete somatostatin, beta cells which make

insulin, delta cells which make glucagon and other cells which make other proteins. To isolate the islet cells which make up only 1-2% of the pancreas from the surrounding acinar tissue, the digestive enzyme collagenase is used. This process is described by Ricordi (*Diabetes* 37:413-410, 1988, hereby incorporated by reference). Once the islets are obtained, they are purified from acinar cells and can then be implanted fresh, cultured for extended periods, cryopreserved indefinitely or encapsulated.

For use in human treatments, primary islet cells are obtained from human cadaver donors or from suitable mammalian sources such as rat, cow, or pig. For use of animal tissue in humans, it is desirable to assure safety of the animal source by using specific pathogen-free (SPF) or gnotobiotic colonies or herds of animals. As an alternative to a primary cell source, an engineered cell line which is genetically altered to produce the proper regulated amounts of insulin, glucagon, somatostatin, etc. is also suitable for treatment of diabetes.

<u>Adrenal chromaffin cells for Parkinson's disease, Alzheimer's and Huntington's disease</u>

Adrenal chromaffin cells have multiple applications. They secrete the neurotransmitter dopamine for amelioration of Parkinson's disease, fibroblast growth factor, and can be engineered to secrete nerve growth factor which will counter degeneration and cell death in Alzheimer's and Huntington's disease. A collagenase digestion method of isolating adrenal chromaffin cells from the adrenal gland is described by Livett (*Physiol. Rev.* 64:1103-1161, 1984). Human or other mammalian sources can be appropriate sources of this tissue.

Moreover, mammalian cells can also be genetically engineered to secrete certain proteins or peptides whose absence or deficiency is the cause of various genetic diseases (i.e. adenosine deaminase deficiency). In addition, such cells can also be engineered to secrete various cytokines and growth factors for the treatment of viral infections (i.e., interferon-γ) and cancer (i.e., interleukin-2). Hormone deficiencies can also be treated by this method. Mammalian cells are transfected with an expression vector containing a gene encoding such a therapeutic protein or peptide. These expression vectors are constructed using standard methods well known to one of

ordinary skill in the art. A tolerizing dose of these cells is encapsulated as described herein and implanted into a mammal. Two to three weeks later, a curative dose of the same cells is implanted into the mammal. The cells are no longer recognized as foreign, are not destroyed by the host immune system and continue to secrete the desired therapeutic protein.

Other conditions treatable by encapsulated cells producing peptides, proteins or other therapeutic agents include hypoparathyroidism (thyroid hormone), hyperadrenocorticalism (adrenocorticotrophic factor), dwarfism (growth hormone), Gaucher's disease (glucocerebrosidase), Tay-Sachs (hexosaminidase A) and cystic fibrosis (cystic fibrosis transmembrane regulator). In addition, cells expressing stimulatory or inhibitory cytokines can be encapsulated, resulting in stimulation or inhibition, respectively, of a particular cell type. For example, erythropoietin stimulates red blood cell production, interleukin-2 stimulates the proliferation of tumor-infiltrating lymphocytes and interferons inhibit certain types of tumor cells. Other conditions contemplated for treatment using the method of the present invention include amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's Chorea, epilepsy, hepatitis, anxiety, stress, pain, addiction, obesity, menopause, endometriosis, osteoporosis, hypercholesterolemia, hypertension and allergies.

Other cell sources and methods for other diseases

Other cell/tissue sources and methods include collagen recovery from chicken for prevention and treatment of arthritis, Schwann cells from myelin tissue for prevention and treatment of neural degeneration and Factor VIII from liver hepatocytes for treatment and prevention of hemophilia.

The amount of cells or tissue necessary for the initial tolerizing implant will vary depending on the disease, site, source, whether the tissue is primary or immortalized and other factors. Generally, the tolerizing dose is one or two orders of magnitude less than a full dose implant. For example, in diabetes it usually takes between about 10,000 - 20,000 islets/kg of body weight to provide adequate insulin production for normoglycemia. Accordingly, the initial implant dose for tolerization is about 100 - 2,000 islets/kilogram of body weight. Although the size of these doses are not known for all disease states, they can be optimized using routine dose/response

experiments well known to one of ordinary skill in the art. In general, between about 100 cells/kg body weight and about 5,000 cells/kg body weight are suitable for tolerization. The corresponding curative doses are between about one and two orders of magnitude higher than these numbers.

Preparation of encapsulation device, loading of cells and implantation

The membrane for the device is chosen for the application needed based on its biocompatability, permeability, strength, durability, ability to be manipulated and other important considerations. A number of materials have already been shown to be acceptable for implants in mammals. Examples of some of these materials are PAN/PVC acrylic co-polymers, hydrogels such as alginate or agarose, mixed esters cellulose, polytetrafluoroethylene (PTFE)/polypropylene (Lum et al., *Diabetes* 40:1511-1516, 1991; Aebischer et al., *Exp. Neurol.*, 111:269-275, 1991; Liu et al., *Hum. Gene Ther.* 4:291-301, 1993; Hill et al., *Cell Transplantation* 1:168, 1992, all hereby incorporated by reference) and polyethylene glycol (PEG) conformal coating configurations (U.S. Patent No. 5,529,914, hereby incorporated by reference).

A critical factor is the pore size that can be produced in the material chosen. For example, PEG macromers can vary in molecular weight from 0.2 - 100 kDa. The degree of polymerization, and the size of the starting macromers, directly affect the porosity of the resulting membrane. Thus, the size of the macromers are selected according to the permeability needs of the membrane. It is believed that for xenograft transplants (animal to human), antibodies of the immune system and complement are involved in rejection (Bachet al. *Transplantation* Overview 6(6):937-947, 1991). In this case, a pore size (molecular weight cutoff) of 150 kDa or smaller is desired to prevent the passage of the smallest immune antibody (IgG) through the pores of the membrane capsule. Thus, the application and its conditions will determine the choice of membrane material from many available alternatives. Likewise, the configurations of the device will be determined by the application. For purposes of encapsulating cells and tissue in a manner which prevents the passage of antibodies across the membrane but allows passage of nutrients essential for cellular metabolism, the preferred starting macromer size is in the range of about 3 kDa to 10

kDa, with the most preferred being about 4 kDa. Smaller macromolecules result in polymer membranes of a higher density with smaller pores.

It is also believed that for allografts (human to human), only entry of immune system cells must be blocked to prevent rejection of transplanted tissue or organs (Auchincloss, Jr., *Transplantation* Overview 46(1):1-20, 1988. In addition, it is also desirable to exclude other cells, the smallest of which are red blood cells which have a size of about 7 μm. Accordingly, a membrane having a molecular weight cutoff of about 150 kDa is also suitable for encapsulation of allograft cells or tissue because such membranes will prevent entry of such cells. In an alternative embodiment, the pore size for allografts is about 0.4 μm or less to prevent the entry of immune and non-immune cells into the device. Cells can also extend processes ("arms") which can enter openings having a size of about 0.2 μm. Thus, in another preferred embodiment, the pore size is about 0.2 μm or less. In a most preferred embodiment, the pore size is as small as possible to exclude entry of detrimental components, but allows cell survival by permitting vital molecules such as nutrients, proteins and oxygen to freely pass through the permselective membrane. A desired pore size may be obtained by adjusting the crosslink density and length of PEG segments by one of ordinary skill in the art without undue experimentation.

If retrieval of the initial implant is unnecessary or undesirable, then a suitable configuration may be microcapsules where only a few or even single cells are each enclosed in separate membranes. Because of the small volume in this case, the microcapsules may simply be injected into one of many sites for the implant. If it is desirable to retrieve or reload the device or larger numbers of cells are necessary, a "macrocapsule" may be constructed wherein many cells are enclosed together inside one membrane. In this case, it has been shown that the environment inside the macrocapsule may need special conditions to allow the cells to survive. For example, an alginate matrix has been used to immobilize islet cells and prevent their aggregation and subsequent central necrosis (Lacy et al., *Science*, 254:1782-1784, 1991).

For other cell types a different environment may be needed. The macrocapsule may be of any shape that is practical. Examples of shapes commonly used by those

skilled in the art are: 1) flat sheet "sandwiches" where two layers of the membrane are top and bottom on the cells and the ends are sealed by heat welding, gluing, or other known means (Fig. 2). This method provides a large surface area for membrane exposure to the host systems and generally short diffusion distances which helps transport substances across the membrane; 2) A tubular membrane formed by co-extrusion or rolling a flat sheet into a tube and sealing the ends (Fig. 3).

The cells can be placed inside the lumen at the same time the membrane is formed if co-extrusion is employed. If the tube is made first, the cells are loaded by syringe or other means and the ends are sealed by heat welding, gluing or other known means. As previously discussed, various matrices may be employed as needed by the enclosed cells. The tubes can be any suitable length and may be joined at the ends (potted) or woven if multiple tubes are used; 3) a spherical shape (Fig. 4) which has a large surface area compared to its volume and is efficient in some applications.

These are only illustrative examples of how membranes may be configured into devices to hold cells. One of ordinary skill in the art will appreciate that many more configurations are possible, thus providing great flexibility for many conceivable applications.

The loaded devices are then implanted into patients in need of therapy. The method of implantation, site and duration are dependent on the disease being treated. For example, in diabetes it is desirable to have the shed antigens processed by the liver. Therefore, implantation in the peritoneum where the portal circulation would carry the antigens directly to the liver (intraportal) is a preferred site. Alternatively, if the dose is a small enough volume (i.e. 10 μl or less), direct injection into the portal vein is preferred. Other implantation sites include under the kidney capsule and subcutaneous implantation.

For Parkinson's disease, the cells should be processed first in the brain. Thus implanting into the interstitial region of the brain is a preferred site. For each site, the method of implantation may be different. For example, intraperitoneal placement of a device for diabetes may be performed by a minimally invasive laparoscopic procedure. To place a device in the brain, the neurosurgeon commonly uses stereotaxic instruments to ensure exact placement. For a subcutaneous implant, a

small incision to allow a trocar to be inserted may be used. For each preferred site, those skilled in the art will recognize the most efficient method of implantation.

Once implanted, the cells are left in place for a period of time during which tolerization will occur. This time period will vary depending on the disease treated, whether an allograft or a xenograft transplant is being used, site of the implant, and other factors. Generally, tolerization requires from a few weeks to a few months. During this time, the transplanted cells constantly shed antigens from their surface. These antigens comprise a variety of small molecules which are constantly being replaced by living cells The antigens can pass freely out of the pores of the membrane and into the recipient at the locations of the implant and eventually into the circulatory system. The immune system immediately recognizes these antigens as "foreign" and initiate its mechanisms to protect the recipient from the intruder. These mechanisms are complex and not completely understood. Generally, it is believed that if the foreign matter is from a closely related species (allogeneic), cells of the immune system play the primary role in the immunological response. These cells include T-cells, macrophages, neutrophils, and natural killer cells which seek out the source of the invasion and destroy the foreign matter. If the foreign matter is a transplanted xenogeneic organ, preformed antibodies cause hyperacute (within minutes) reaction and rejection of the organ. If the foreign matter is xenogeneic cells or tissue, the antigen may not be presented and the preformed antibodies may not be the primary mechanism of rejection. Instead, macrophages stimulate killer T-lymphocytes and later (8-10 days) antibody stimulation causes final rejection of cells or tissue.

In the present invention, however, neither system can destroy the cells of the implant when the pore size of the membrane is properly selected for the application. For example, if allografts are destroyed by immune cells, then the membrane pores must only prevent entry of these cells and thus may be about 0.4 μm or smaller. Likewise, if it is necessary to prevent antibodies from reaching the cells, the pores must be smaller than the smallest of the human antibodies, IgG, which is 150 kDa. Of course, a pore size having a molecular weight cutoff of about 150 kDa or less is suitable for tolerization in both allografts and xenografts.

The use of a permselective membrane prevents the immune system from destroying cells encapsulated therein, even though the immune system recognizes the implant tissue as foreign and mounts a classical response. The immune response cannot destroy the cells because they are protected within the membrane device. Because the immune system cannot destroy the cells even over time, the system will come to tolerate the implant and cease trying to destroy it. While the mechanism for this tolerization is not known, it is analogous to desensitizing patients to allergic immune reactions (i.e. antibiotics or bee stings). In fact, an alternative method to the single tolerizing implant is the addition of more cells with more implants over time if necessary. At this point, the immune system basically recognizes this cellular material as "self" and no longer mounts an immune response against it.

Implantation of full curative dose

When the patient has been tolerized to the cells of the implant, a full curative curative size dose of the tissue or whole organ is administered as described in the following examples.

Whole organ transplants - allografts

In one embodiment, the method is used for a human allograft. In this embodiment, the tissue for the initial implant is taken from a living related kidney donor by biopsy or similar method and a tolerizing dose is implanted into the patient. When the patient is tolerized, the whole kidney is taken from the donor and transplanted into the recipient. The graft is accepted with no continuous immunosuppression being necessary.

Whole organ transplants - xenografts

For most embodiments, it is preferable to use animal organs for human transplants. In these embodiments, the procedure is as follows: suitable animal donors are identified. Sources of these donors may be genetically identical (inbred). Tolerizing cells are taken from any animal in the colony. Later, the whole organ is taken from any other animal in the colony. It is preferable that these sources are free of all contaminants of risk to humans so they would preferably be specific pathogens free (SPF) or gnotobiotic (totally isolated in sterile conditions) colonies or herds. Heart, lungs, livers, kidneys, pancreases and other organs may be used in this

embodiment, thus eliminating the critical shortage of these organs from the limited number of available human organ donors.

Cellular transplants - allografts

In this embodiment, the method is used for human to human cellular transplants. A full size therapeutic dose is obtained from the cadaver donor source as previously described. For example, islet cells are obtained from the pancreas of a human donor. The small amount needed for the tolerizing implant is taken from the preparation, encapsulated and implanted as previously described. The remainder of the cells are cryopreserved by known methods (Kneteman et al, *Transplant. Proc.* 18:182-185, 1986) and are held until tolerization is completed. The full preparation is then thawed and ready for implantation. If, in this embodiment, it is necessary to acquire cells from more than one donor to have enough for a curative implant, then the cells for the initial implant are taken from multiple donors and mixed for the implant. The recipient is therefore tolerized to all of the cells from the multiple donors.

Cellular transplants - xenografts

As with whole organs, the present method allows the use of cellular transplants from animals as well. Cells for the initial implant are taken from genetically identical animals or multiple pooled animals as previously described. When the individual is ready for the full transplant, cells may be taken from any other member of the genetically identical colony or from multiple pooled animals if necessary for sufficient curative quantities.

The implantation procedure for the fully curative dose of cells, whether allograft or xenograft, is dependent on the disease, the quantity of cells, the site, and other factors. For example, for diabetes, a preferred procedure for the implantation of islet cells in humans is to inject the cells through the portal vein so that they become lodged in lobes of the liver. This procedure is done under local anesthesia and is minimally invasive to the patient. For treatment of neural disorders, cells can be implanted into any selected area of the brain by well known stereotaxic surgical procedures. Those skilled in the art will know preferred methods for cellular implantation for each embodiment.

Implants for Prevention of Diseases

Identification of patient populations is dependent on the ability to diagnose patients at high risk of developing certain diseases or those in early stages of the disease. Rapid progress has been made in this area of medicine primarily due to major advances in understanding and mapping the human genome. In addition, DNA amplification methods, notably the polymerase chain reaction (PCR), can be used to diagnose certain genetic disorders. Other research areas for predicting diseases are advancing as well.

In diabetes, the use of immune marker autoantibodies to establish preclinical diabetes has been well studied (Palmer, *Diabetes Rev.* 1(1):104-116, 1993). When these patients are identified, the physician determines at what point in the course of the disease it would be most advantageous to intervene.

Individuals determined to be at risk for development of a particular disease are implanted with the appropriate cell type as described above. Methods for acquiring small amount of cellular tissue for the initial tolerizing implant, tissue types, the amount of tissue necessary for implantation, preparation of the encapsulation device, loading cells into the device, implanting the device into a patient, membrane parameters, device configuration, implantation methods, curative dose administration, etc. are the same as discussed hereinabove for disease treatment.

Treatment of Diseases Arising from Lack of a Hormone

A study was performed using an insulin-producing mouse tumor cell line encapsulated in a permselective membrane coating as described in the following example.

Example 1

Implantation of mouse insulinoma cells

The NIT insulin-producing mouse tumor cell line was encapsulated with PEG conformal coatings of a single configuration, 11% PEG 4,000 kDa molecular weight (See U.S. Patent No. 5,529,914), which corresponds to a molecular weight cutoff of between about 10 kDa and about 70 kDa. The encapsulated cells were implanted beneath the kidney capsule at two different doses into C57B6 mice of a different allograft haplotype in which diabetes had been induced by intravenous injection (tail

vein) of 167 mg/kg body weight of streptozotocin (Upjohn, Kalamazoo, MI). Induction of diabetes by streptozotocin injection is a well known procedure which destroys pancreatic insulin-producing β cells.

Tolerizing doses of encapsulated insulinoma cells were 50 or 100 cell aggregates, each containing about 1,000 cells. Encapsulated cells were implanted beneath the kidney capsule using standard surgical procedures. Curative implants of unencapsulated insulinoma cells (2,000 - 3,000 insulinoma cell aggregates, each containing about 1,000 cells) were administered by free intraperitoneal injection 15 or 20 days after the tolerizing dose to determine whether a sufficient quantity of cells survived. Control animals were given only the curative dose of insulinoma cells. Blood glucose levels were monitored and are shown for the 100 encapsulated NIT aggregate tolerizing dose, 50 encapsulated NIT aggregate tolerizing dose and non-tolerized controls (Figures 5, 6 and 7, respectively).

The severity of streptozotocin-induced diabetes in these mice caused several of the animals to die during the periods of observation and during procedures done as part of the study. Table 1 indicates the number of animals involved in the study and their outcomes. The degree of diabetes is very high, with values over 500 mg/dl (shown as 500) for all streptozotocin-induced animals in the study. Many of these severely diabetic animals died of their diabetes during the study or following a procedure as noted. As shown in Figure 5, of the first group of 8 diabetic mice receiving 100 encapsulated aggregates, only four survived for the challenge 20 days later with the unencapsulated aggregates. Two of these died overnight following the IP injection. The remaining two recipients both had a sudden and marked reduction in their glucose values between 5 and 9 days, with glucose values reaching levels of 40 mg/dl and below (BM5 and BM11, Figure 5 and Table 1). If the insulin-secreting insulinoma cells induce immunological tolerance, the curative implant will be recognized as "self" and will not be destroyed by the recipient's immune system. Because the NIT cells are tumor cells which double every 2-3 days *in vitro*, their survival would be expected to result in recipient hypoglycemia due to the increasing insulin-producing cell mass that would occur from living and growing tumor cells.

In the second group of three recipients of 50 encapsulated aggregates for 15 days, two died of their diabetes prior to challenge with unencapsulated NIT cells. The one animal that received the challenge of unencapsulated NIT cells (BM16) has not exhibited any reduction in blood glucose values for the same time of observation (Figure 6). None of the control animals only challenged with unencapsulated NIT cells exhibited any reduction in blood glucose values (Figure 7).

The results indicate that encapsulated NIT cells given as a small mass prior to a large, unencapsulated curative cell implant permits the second curative dose to survive, reducing blood glucose values in a pattern suggestive of NIT tumor cell growth. A smaller dose of encapsulated NIT cells did not give this result. Control animals that only received unencapsulated NIT cells in a curative dose exhibited no reduction in blood glucose. These results indicate that the preliminary encapsulated implant tolerized the host to the following unencapsulated curative dose. When such a preliminary encapsulated implant was not done, the curative unencapsulated implants had no effect on blood glucose and were presumably destroyed by the host.

Table 1

Animal #	Toler. Encap. Implant	# Encap. Cell Agg. Tol. Dose	Delay to Cure Implant	Unencap. Cell Implant	# Unenc. Cell Agg. Cure	Effect on Blood Glucose
BM1	yes	100	20 days	yes	2348	none-died*
BM3	yes	100	20 days	no-died	-	n/a
BM4	yes	100	19 days	no-died	-	n/a
BM5	yes	100	20 days	yes	2348	down to 40
BM6	yes	100	20 days	no-died	-	n/a
BM7	yes	100	20 days	yes	2348	none-died*
BM10	yes	100	20 days	no-died	-	n/a
BM11	yes	100	19 days	yes	2348	down to 40
BM14	yes	50	15 days	no-died	-	n/a
BM15	yes	50	15 days	no-died	-	n/a
BM16	yes	50	15 days	yes	2348	none-500

Animal #	Toler. Encap. Implant	# Encap. Cell Agg. Tol. Dose	Delay to Cure Implant	Unencap. Cell Implant	# Unenc. Cell Agg. Cure	Effect on Blood Glucose
BM29	no	0	-	yes	2352	none-500
BM31	no	0	-	yes	2352	none-500
BM32	no	0	-	yes	2352	none-500
BM34	no	0	-	yes	2352	none-500
BM35	no	0	-	yes	2352	none-died*
BM36	no	0	-	yes	2352	none-500
BM40	no	0	-	yes	2352	none-500
BM41	no	0	-	yes	2352	none-died*
BM42	no	0	-	yes	2352	none-500
BM43	no	0	-	yes	2352	none-died*
BM44	no	0	-	yes	2352	none-500
BM45	no	0	-	yes	2352	none-500

* Died during course of experiment-no effect on blood glucose

Example 2

Use of encapsulated islets for induction of allograft tolerance in rats

Rat pancreatic islet cells are isolated by a standard collagenase digestion method (Ricordi, *Diabetes* 37:413-410, 1988) and cultured for three days prior to PEG encapsulation. Donor islets are derived from the Wistar Furth (WF) strain having MHC haplotype RT1-U. Recipients are of the Lewis strain having MHC haplotype RT1-1. Transplants across this strain combination are normally rejected within three weeks. Islet transplant mass is dosed on the basis of a standard 150 μm diameter rat islet; an Islet Equivalent (Ieq). Islets are quantified and tested for sterility and mycoplasma prior to encapsulation and implantation.

Islet cells are conformally coated with 11% PEG 4,000 kDa molecular weight by the method described in U.S. patent No. 5,529,914. As a negative control, acellular cross-linked dextran beads are encapsulated in a similar manner. Diabetes is induced in fasted Lewis rats by intravenous injection of streptozotocin (65 mg/kg) one week prior to implantation of the tolerizing dose and monitored during that week for blood glucose levels and weight changes. Rats are considered diabetic once their

blood glucose level exceeds 350 mg/dl. Rats having a minimal weight loss and blood glucose levels of 300-350 mg/dl are used for the study.

Diabetic rats are implanted by trochar with a subcutaneous 30 day time release depot insulin (Linplant, Lishin, Ontario, Canada) to reduce the chances of ketosis/acidosis and to stabilize their diabetes. Animals remain hyperglycemic at this Linplant dose (2 units of bovine insulin in 24 hours - lasts 30 days).

Diabetic MHC disparate Lewis rats are surgically implanted once with encapsulated donor WF islets at the renal subcapsular site after anesthetization. The dose of implanted cells varies as outlined in Table 2.

Table 2

Group	N	Dose	Rationale
1	12	1200 encap islets	high dose sensitization/tolerization
2	12	600 encap islets	low dose tolerization
3	12	300 encap islets	very low dose tolerization
4	12	1200 encap acellular beads	control for polymer

As a control, a set of recipients (Group 4) is implanted with encapsulated acellular beads to control for possible polymer effects in tolerization. All implanted animals are maintained for intervals as shown in Table 3 prior to the second transplantation. At the time of implantation, serum samples from each animal are drawn and retained for future immunological analysis.

Table 3

Group	N	Dose	Implant Interval (days)	Rationale
1a	4	1200 encap islets	30	high dose sensitize/tolerize-short interval
1b	4	1200 encap islets	60	high dose sensitize/tolerize-intermediate interval
1c	4	1200 encap islets	90	high dose sensitize/tolerize-long interval
2a	4	600 encap islets	30	low dose tolerization-short interval
2b	4	600 encap islets	60	low dose tolerization-intermediate interval
2c	4	600 encap islets	90	low dose tolerization-long interval
3a	4	300 encap islets	30	very low dose tolerization-short interval
3b	4	300 encap islets	60	very low dose tolerization-intermediate interval
3c	4	300 encap islets	90	very low dose tolerization-long interval

4a	4	1200 encap acell-beads	30	polymer control-short interval
4b	4	1200 encap acell-beads	60	polymer control-intermediate interval
4c	4	1200 encap acell-beads	90	polymer control-long interval

During the indicated period, animals are monitored for weight changes and blood glucose levels. One week before the second transplant, one animal in each of Groups 1a-1c, 2a-2c, 3a-3c and 4a-4c is sacrificed and the implant site analyzed by histological methods for determining viability of the tolerizing cells.

Lewis rats remaining in Groups 1-4 receive a second transplant (curative dose) of WF islets which are unencapsulated. Transplant sites in each animal are intraportal (IP) at a dose of 6,000 Ieq and at one kidney with a dose of 100 Ieq (See Table 4). 6,000 Ieq implanted into the liver is known to be a curative dose in the rat diabetes model. The 100 Ieq kidney capsule implant is only for histology at the end of the experiment. At the time of the second implant, serum samples from each animal are drawn and retained for future immunological analysis. For the next three weeks, animals are monitored for blood glucose levels and weight changes. At the termination of the experiment, graft sites are processed for histology. At this time, serum samples from each animal are again drawn and retained for future immunological analysis.

Table 4

Group	N	Recipient haplotype	Dose # of islets	Implant Sites	Duration of Transplant
1a	3	RT1-1	6000	IP/kidney	3 weeks
1b	3	RT1-1	6000	IP/kidney	3 weeks
1c	3	RT1-1	6000	IP/kidney	3 weeks
2a	3	RT1-1	6000	IP/kidney	3 weeks
2b	3	RT1-1	6000	IP/kidney	3 weeks
2c	3	RT1-1	6000	IP/kidney	3 weeks
3a	3	RT1-1	6000	IP/kidney	3 weeks
3b	3	RT1-1	6000	IP/kidney	3 weeks
3c	3	RT1-1	6000	IP/kidney	3 weeks

4a	3	RT1-1	6000	IP/kidney	3 weeks
4b	3	RT1-1	6000	IP/kidney	3 weeks
4c	3	RT1-1	6000	IP/kidney	3 weeks

In Groups 1 and 4, no changes in the diabetic state are measured. In Group 4, rejection occurs in the expected two week time frame as measured by a transient normoglycemia followed by a return to the diabetic state. In Group I, a more rapid rejection of the implant due to sensitization of the recipients occurs. In the recipients previously exposed to tolerizing doses of encapsulated WF islets (Groups 2 and 3), islet cells survive and result in a continuous maintenance of normoglycemia.

Example 3

Use of encapsulated islets for induction of allograft tolerance in humans

Human islets are isolated from cadavers and 1,500 islets/kg body weight are PEG-encapsulated and implanted under the kidney capsule in a diabetic patient. After two months, a curative dose of 15,000 unencapsulated islets/kg body weight are injected intraportally. Insulin administration is continued during the course of the protocol up to administration of the curative dose. Blood glucose levels are constantly monitored and are within the normal range.

Example 4

Treatment of Parkinson's disease (xenograft)

Adrenal chromaffin cells are isolated from inbred baboon adrenal glands and 1,000 cells/kg body weight are encapsulated in an appropriate PEG conformal coating. The capsule is implanted into the interstitial brain region of a human by a neurosurgeon using stereotaxic instruments. After 1 month of tolerization, 10,000 unencapsulated cells/kg body weight are injected into the same brain region. Significant improvement in the condition is observed.

Example 5

Prevention of hemophilia

A male individual at risk of developing hemophilia, an x-linked disorder, by virtue of family history, is subjected to genetic screening to determine the presence or absence of the gene encoding Factor VIII, and to clotting time analysis. If the gene

is absent or clotting time is reduced, 2,500 liver cells/kg recipient body weight are isolated from a human donor and encapsulated in a PEG conformal coating. The encapsulated cells are implanted under the kidney capsule. One month later, 5,000 cryopreserved liver cells/kg recipient body weight (from the same donor) are injected intraportally. Clotting time is significantly improved.

Example 6

Liver transplant (xenograft)

An individual in need of a liver transplant is subcutaneously implanted with 1,000 PEG-encapsulated liver cells/kg body weight isolated from an inbred baboon. Two months later, the entire liver is transplanted into the individual. Signs of organ rejection and vital signs are monitored over several months. Rejection does not occur.

Example 7

Prevention of myasthenia gravis (xenograft)

Myasthenia gravis is an autoimmune disorder resulting from the presence of antibodies against the acetylcholine receptor on neurons. An individual having very early signs of the disease is implanted under the kidney capsule with a tolerizing dose of 2,500 PEG-encapsulated neural cells/kg recipient body weight expressing the acetylcholine receptor isolated from baboons. This results in tolerization to the acetylcholine receptor and prevention of the disorder.

It should be noted that the present invention is not limited to only those embodiments described in the Detailed Description. Any embodiment which retains the spirit of the present invention should be considered to be within its scope. However, the invention is only limited by the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of creating immunological tolerance to foreign cells, tissues or organs in a mammal, comprising the step of implanting in said mammal a tolerizing dose of corresponding foreign cells or tissue which shed antigens contained in or on said foreign cells tissues or organs, said corresponding foreign cells or tissue being encapsulated in a biologically-compatible permselective membrane.

INDUCTION OF IMMUNOLOGICAL TOLERANCE

Abstract of the Invention

A method of creating tolerance to transplanted cells, tissue, or organs without the need for continuous immunosuppression. A tolerizing dose of a cell or tissue within a membrane structure is implanted into a patient. Once the patient becomes tolerant to the cell or tissue, a tissue or organ is implanted which will no longer be recognized as foreign matter. The method makes animal organs practical for human use, prevents autoimmune destruction as well as immune rejection. It has applications in treatment and prevention of many mammalian diseases.

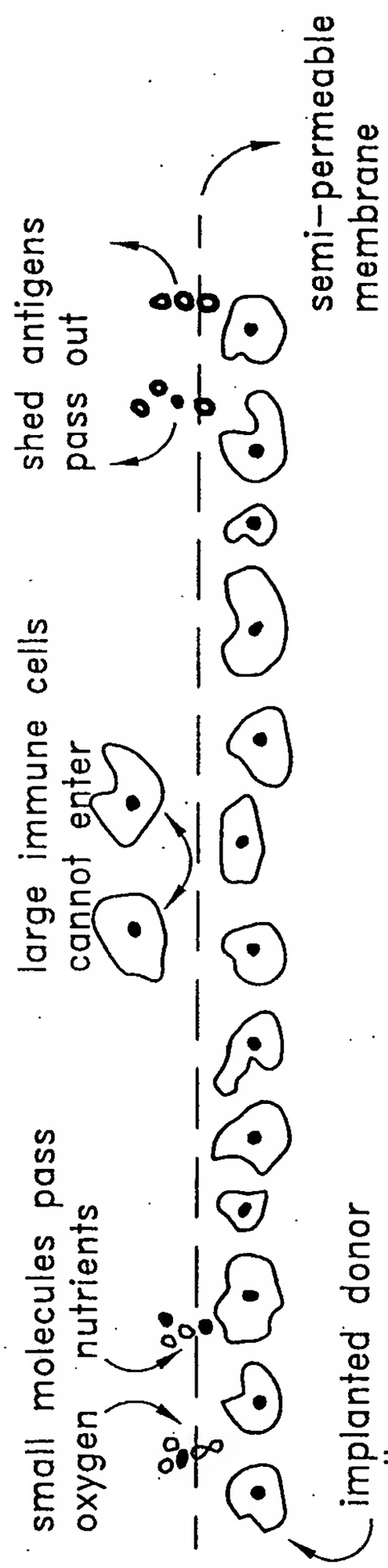
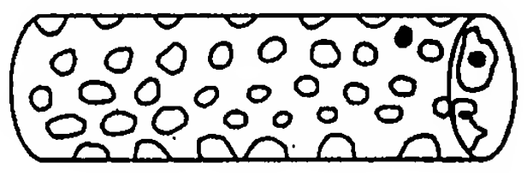

large immune cells cannot enter
shed antigens pass out
semi-permeable membrane
small molecules pass oxygen nutrients
implanted donor cells

FIG. 1

flat sheet

FIG. 2



tubular

FIG. 3

spherical

FIG. 4

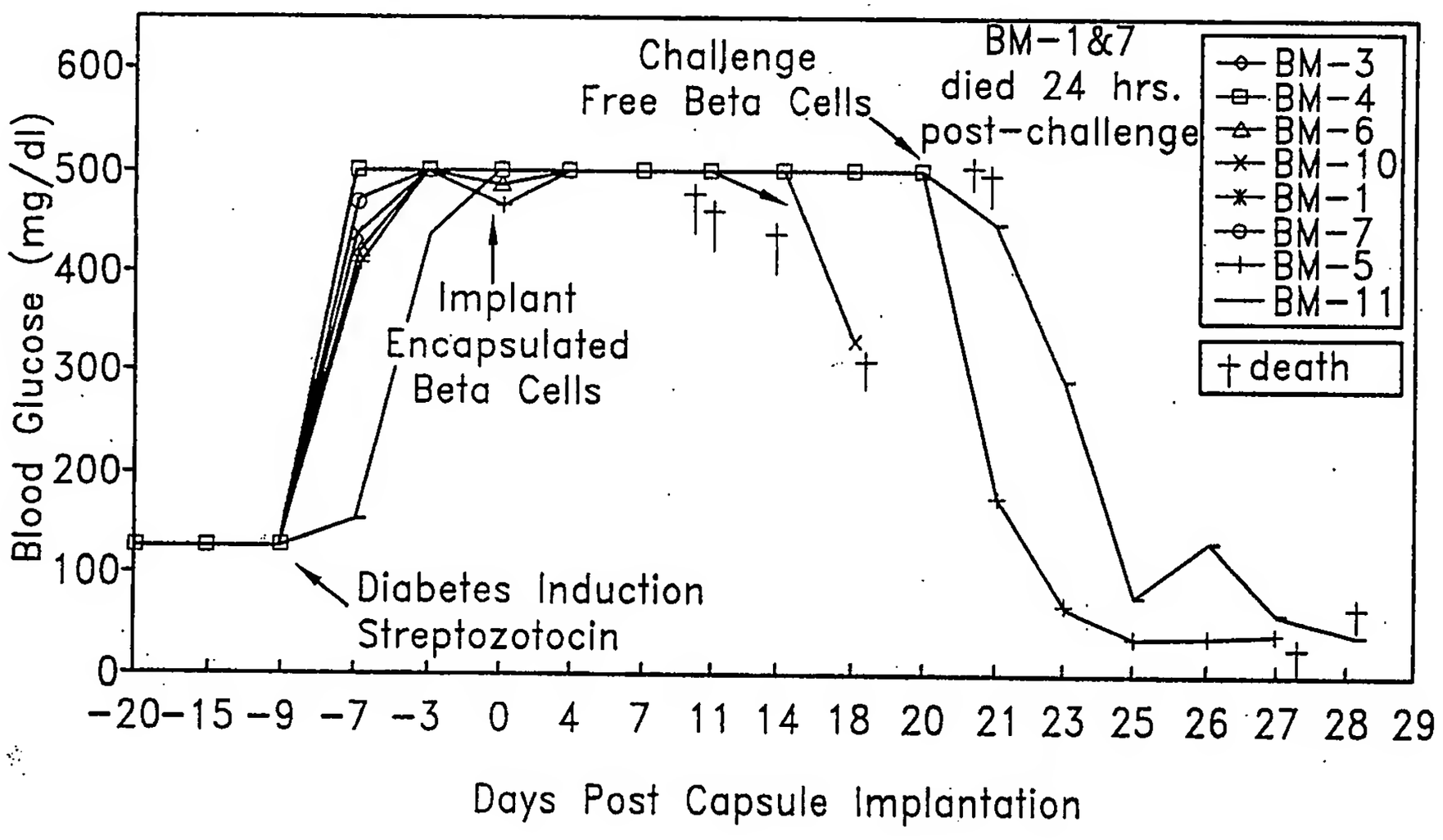

Challenge
Free Beta Cells
BM-1&7
died 24 hrs.
post-challenge
BM-3
BM-4
BM-6
BM-10
BM-1
BM-7
BM-5
BM-11
†death
Implant
Encapsulated
Beta Cells
Diabetes Induction
Streptozotocin
Blood Glucose (mg/dl)
600
500
400
300
200
100
0
-20 -15 -9 -7 -3 0 4 7 11 14 18 20 21 23 25 26 27 28 29
Days Post Capsule Implantation

FIG. 5

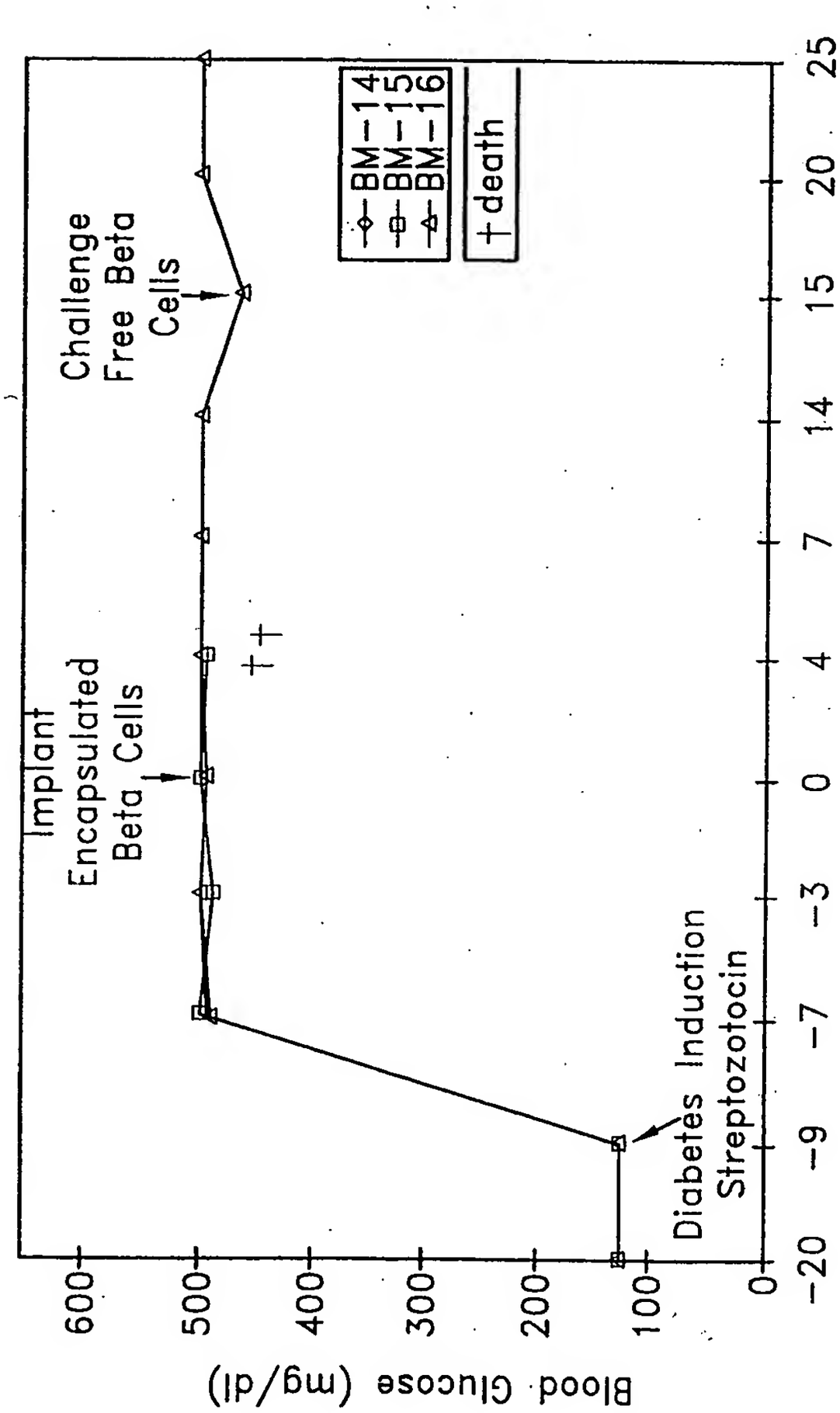

Blood Glucose (mg/dl)
600
500
400
300
200
100
0
Implant Encapsulated Beta Cells
Challenge Free Beta Cells
Diabetes Induction Streptozotocin
++
BM-14
BM-15
BM-16
+death
-20
-9
-7
0
4
7
14
15
20
25
Days Post Capsule Implantation

FIG. 6

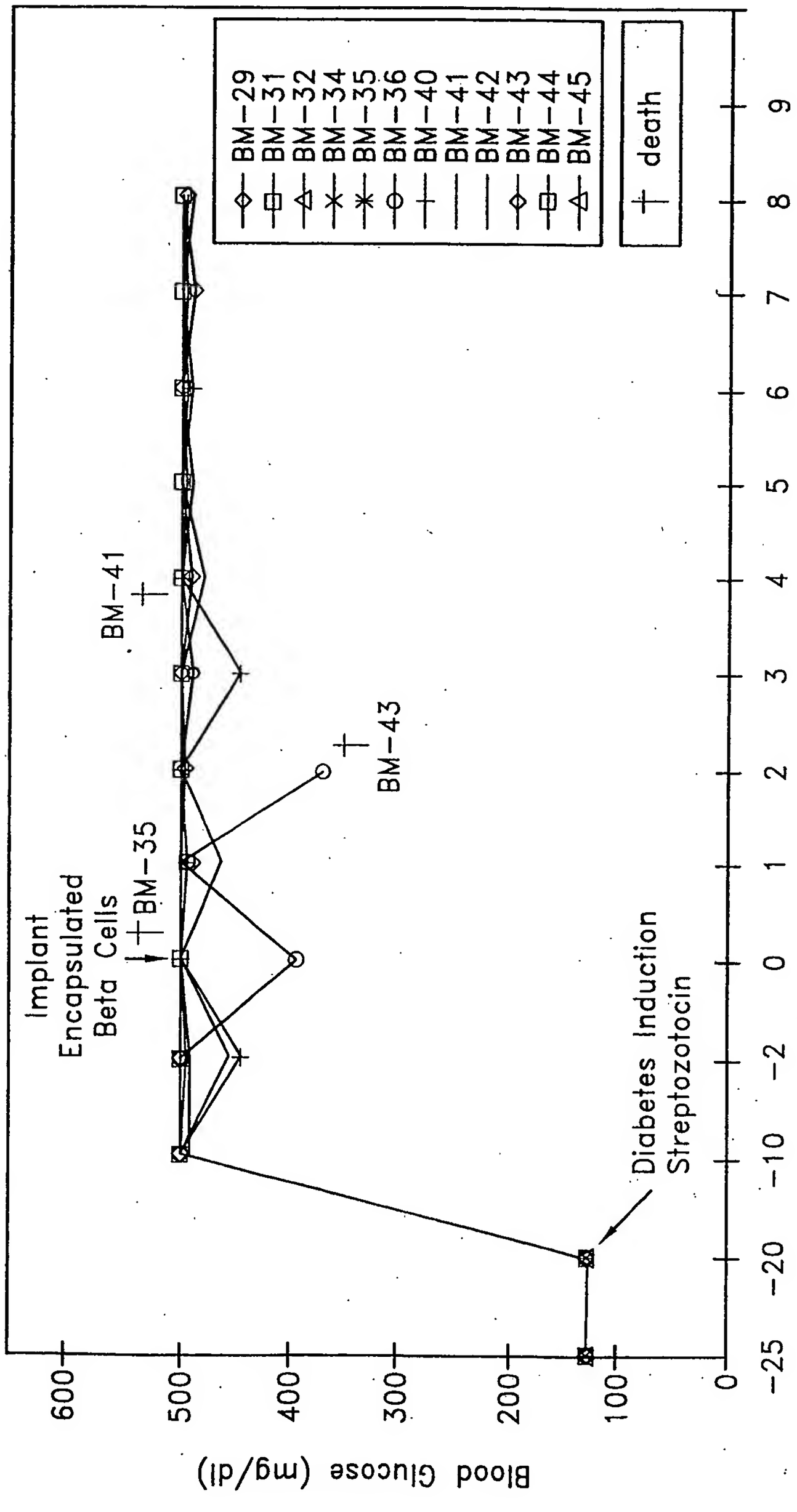

Blood Glucose (mg/dl)
600
500
400
300
200
100
0
-25
-20
-10
-2
0
1
2
3
4
5
6
7
8
9
Days Post Capsule Implantation
Diabetes Induction
Streptozotocin
Implant
Encapsulated
Beta Cells
BM-35
BM-41
BM-43
death
BM-29
BM-31
BM-32
BM-34
BM-35
BM-36
BM-40
BM-41
BM-42
BM-43
BM-44
BM-45

FIG. 7

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Paul P. Latta
App. No.	:	unknown
Filed	:	September 12, 2003
For	:	PREVENTION OF DIABETES THROUGH INDUCTION OF IMMUNOLOGICAL TOLERANCE (as amended)
Examiner	:	Schwadron

DECLARATION OF DAVID SCHARP, M.D.

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
Dear Sir:

1. I, David Scharp, M.D., am Chief Scientific Officer of Novocell, Inc. At Novocell, I am actively engaged in research related to development of treatment of diabetes using encapsulated insulin-producing cells.

2. Novocell, Inc. is a mid-stage biopharmaceutical company developing a unique encapsulated cell implantation technology for the treatment of diabetes and other serious diseases and disorders. The inventor of the above-captioned application is the president of Novocell. Novocell has an option to license the technology disclosed and claimed in the present application.

3. I have over 30 years of experience working in the area of cell biology. A copy of my C.V. is attached as Exhibit A.

4. Working with the sole inventor of the present application, Paul P. Latta, and others, I carried out experiments to evaluate the efficacy of prevention of diabetes using encapsulated islet cells, as described in the specification of the present application.

5. To evaluate the efficacy of these techniques, I developed a protocol for prevention of diabetes in NOD mice using encapsulated murine islets. The objective was to implant

encapsulated C57B6 islets into diabetes prone NOD mice at different doses, and at different times, prior to the normal onset of spontaneous diabetes, to see if immune destruction of NOD mouse islets could be prevented.

6. For purposes of the experiments described herein a mouse is defined as being diabetic when its blood glucose level was >250 mg/dl on three consecutive occasions. Typically, spontaneous autoimmune diabetes has developed in 80% of NOD mice by 112 – 140 days of age.

7. Islets from mouse strain C57B6 were encapsulated by PEG conformal coating as described in U.S. Patent No. 5,529,914. This patent was incorporated by reference into the specification of the application captioned above at page 13, line 15. The conformally-coated islets were implanted by intraperitoneal injection into NOD mice. C57B6 islets contain an MHC mismatch to the NOD strain. Thus, in the absence of conformal coating, rejection of the islets would be expected.

8. NOD mice were divided into groups with two different variables: (a) time of implant of encapsulated islets (4, 8 or 12 weeks of age) and (b) dose of islets (untreated controls and 50, 100 or 150 encapsulated islets). The mice were followed for 250 days.

9. The results are shown in the attached figures. The figures show bar graphs for the 10 treated mice and 3 untreated mice assigned to each time of implantation group. Mice that remained diabetes-free after 250 days are shown with red bars. One mouse treated with 100 islet equivalents (IEQ) at 12 weeks of age remained diabetes-free until 249 days of age, and is therefore shown with a blue bar.

10. It can be seen that all 9 of the untreated controls developed diabetes prior to 200 days. In contrast, many of the mice treated with encapsulated islets remained diabetes-free after 250 days. In particular, 6 out of the 10 mice treated with encapsulated islets at 4 weeks of age; 5 out of the 10 mice treated with encapsulated islets at 6 weeks of age; and 3 out of the 10 mice treated with encapsulated islets at 8 weeks of age remained diabetes-free. The diabetes-free mice were followed for an additional 50 days, and many of these mice were still diabetes-free at age 300 days.

11. The foregoing results demonstrate that Type I diabetes in a mammal predisposed to develop diabetes can be prevented by implanting insulin-producing cells that are encapsulated in a biologically-compatible membrane, when administered to the mammal prior to clinical onset of Type I diabetes.

12. These series of experiments, and the knowledge of one skilled in the art, demonstrate what is important in developing a method to alter the autoimmunity to prevent ongoing destruction of affected tissues.

13. It is important that the cells are encapsulated, but not the method of encapsulation [macrodevice or microdevice, or macroencapsulation, microencapsulation, microcapsules or conformal coating] or the material used for encapsulation [alginate, PEG, PEG/alginate, agarose, or any of numerous other materials]. It is the physical barrier surrounding the cells that allows the nutrients and oxygen to enter the cell and the antigens from the encapsulated cells to enter the blood, while at the same time the physical barrier prevents the immune system from killing the cells producing the immunological response. Additionally, the encapsulation allows the continuous, chronic release of antigens from the cells to effect the correct response from the immune system to stop the autoimmune destruction.

14. It is important, in relation to the onset of the autoimmune disease, when and how many encapsulated cells are transplanted into the animal, but not where the transplantation site is located in the body. I believe it is the timing of transplantation and amount of cells that stops the autoimmune destruction, and not whether the transplantation site is subcutaneous, intramuscular, intraorgan, arterial/venous vascularity of an organ, cerebro-spinal fluid, or lymphatic fluid, since all allow interaction between the encapsulated cells and the immune system.

15. It is important that the cells, which are encapsulated, release antigens specific to the autoimmune response against which the autoimmune destruction is focused, but not that the cells be a specific type or from a specific organ.

16. It is important that it is an autoimmune disease for which immune protection is sought, but not the specific autoimmune disease since there is a common mechanism by which protection from autoimmune destruction is developed in the body.

17. The method of preventing autoimmune destruction by the chronic exposure of the mammal to antigens, which elicit an autoimmune response, that are continuously released from the encapsulated cells takes advantage of two important regulatory functions in the immune system to determine "self" and "non-self". The invention induces a self tolerance using the body's natural "self-tolerance" mechanism. Autoimmune diseases are caused by a breakdown in the normal self recognition process of the immune system. Correcting this breakdown, well

before serious damage is done to the body alleviates the autoimmune disease and prevents the eventual tissue damage.

18. The critical aspect of the invention is correcting this breakdown after it has begun but prior to irreversible damage has occurred, and thus promoting the re-establishment of self acceptance to the antigen.

19. An autoimmune disease is not, *per se*, a disease in the classical sense caused by bacteria or viri. Autoimmunity is defined as breakdown of mechanisms responsible for self tolerance and induction of an immune response against components of the self. The immune system incorrectly identifies self proteins as foreign and mounts an attack against the body's tissue. Left unchecked, the immune response continues to accelerate until it destroys the tissue releasing the antigen and causes serious metabolic problems or death. Both antibodies and effector T cells can be involved in the damage in autoimmune diseases.

20. The exact etiology of autoimmune diseases is not known. However, various theories have been offered. These include sequestered antigen, escape of auto-reactive clones, loss of suppressor cells, cross reactive antigens including exogenous antigens (pathogens) and altered self antigens (chemical and viral infections). Some of these may also be on a background of gene expression that makes the destruction easier.

21. Although the agent that causes the immune system to make the initial error in the recognition of self and begins the onset of the autoimmune disease may be different for each syndrome, the mechanisms of the immune system in the body are the same. Therefore, a method which reverses the unchecked immune response and re-establishes tolerance to "self", will have an universal effectiveness on autoimmune diseases, no matter what caused the error in the first place.

22. Therefore, Type I diabetes can be considered a "Model disease" for autoimmune diseases in general, since the development of autoimmune diseases have a similar pathway, whether it is Type I diabetes, MS, Lupus or any of the other autoimmune diseases. Therefore, a method developed to reverse the onset of Type I diabetes will have universal application to other autoimmune diseases.

23. Preventing or stopping autoimmune destruction is quite different from non-specific immunosuppression and immunodeficiency. It is an active antigen-dependent process in response to the antigen. Like an immune response, eliminating autoimmune destruction is

specific and like immunological memory, it can exist in T-cells, B cells or both and like immunological memory, the T cell effect is longer lasting than the B cell effect.

24. The final achievement in preventing autoimmune destruction in humans by the chronic exposure to self-proteins from encapsulated cells may be due to the priming cells being unable to give a second signal to host T cells with the immune effect maintained by the continued presence of the native cells in the recipient.

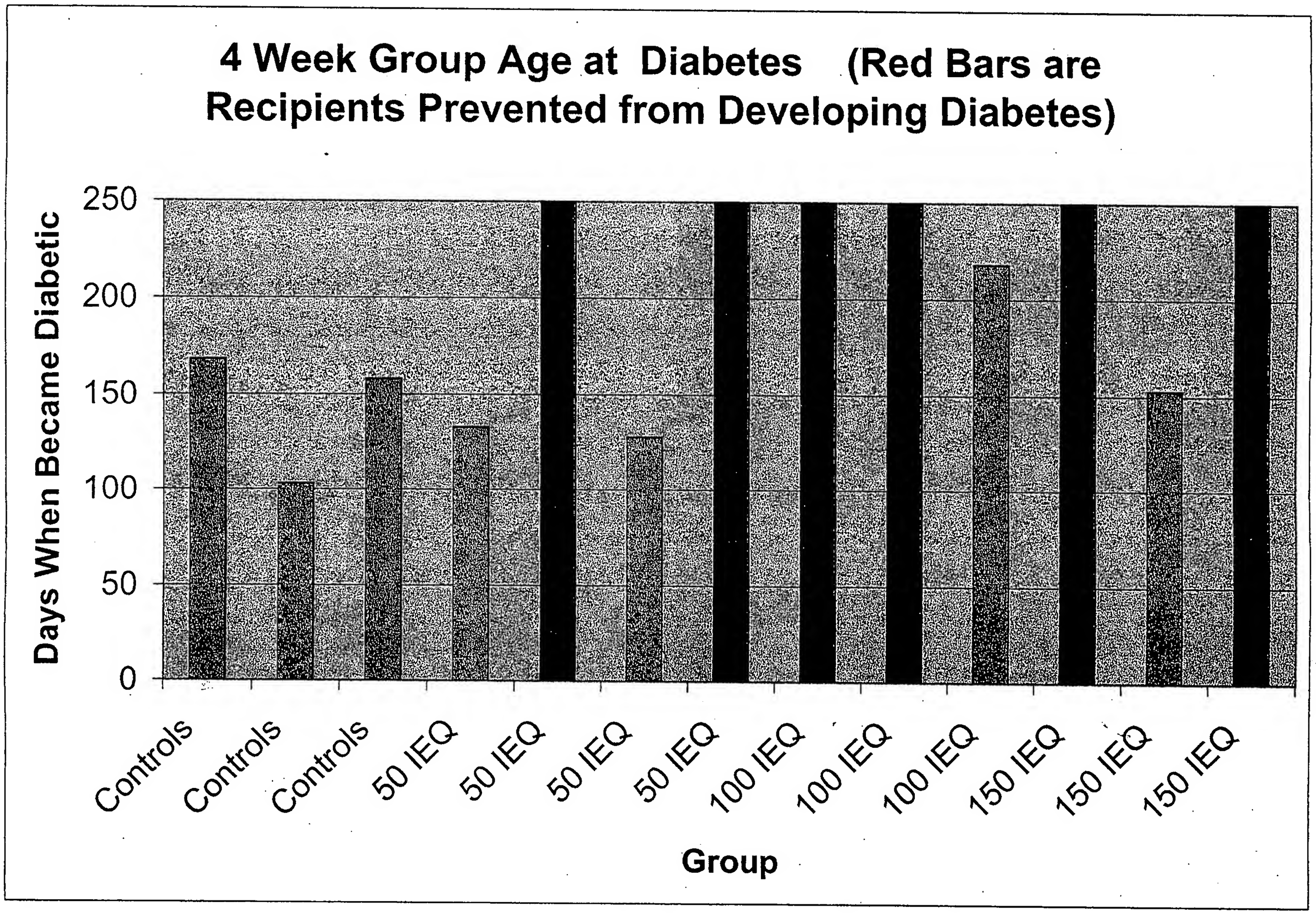
25. The current invention takes advantage of the body's own immune system to generate "self-tolerance" to an antigen(s) it previously mistook as "foreign", which initiated the autoimmune disease cascade. It is this unique manipulation of the body's own immune system that corrects the previous error of the immune system.

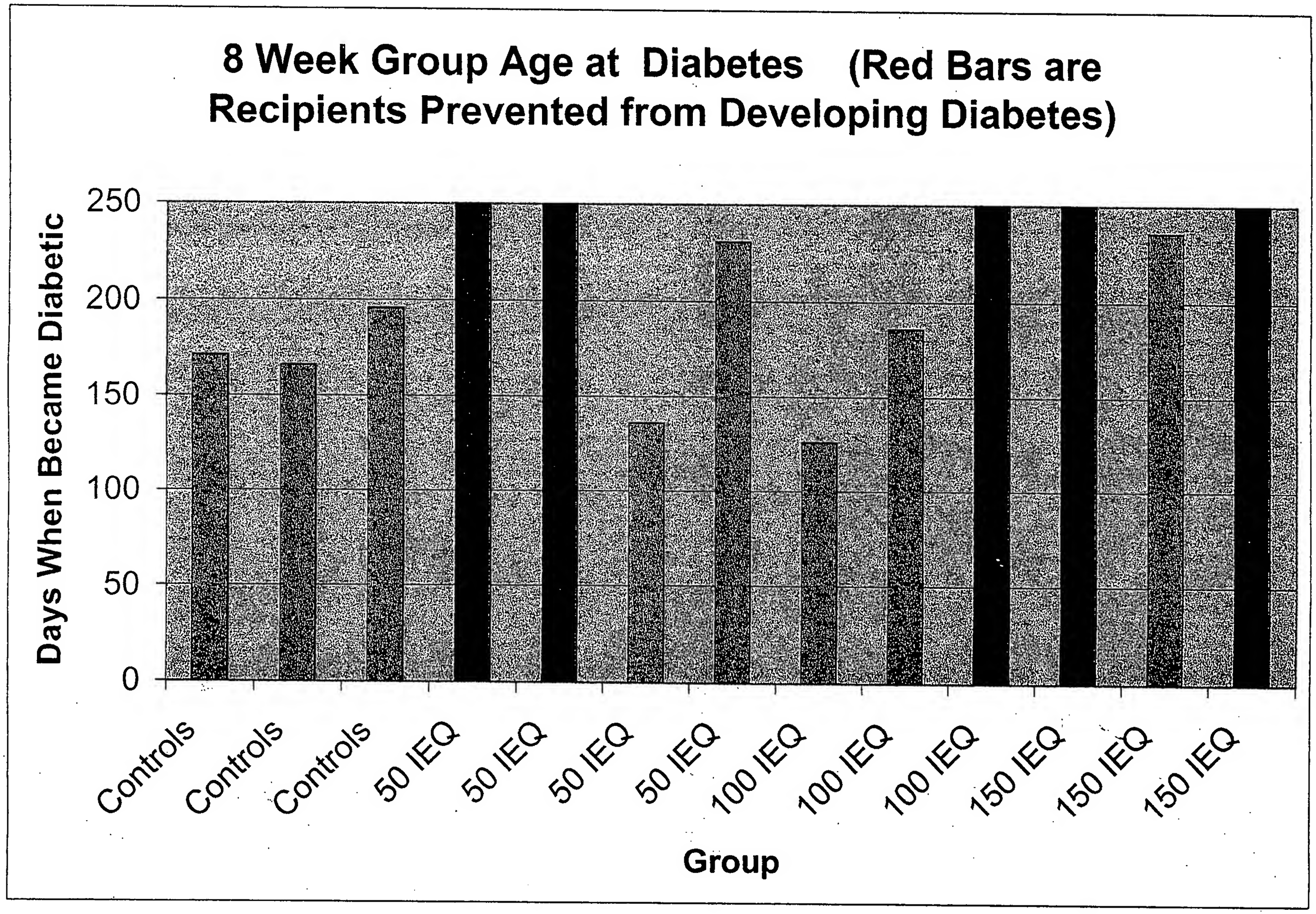
Respectfully submitted,

Dated: 11-25-03

By: David W Scharp

David Scharp, M.D.
Chief Scientific Officer
Novocell, Inc


4 Week Group Age at Diabetes (Red Bars are Recipients Prevented from Developing Diabetes)
Days When Became Diabetic
250
200
150
100
50
0
Controls
Controls
Controls
50 IEQ
50 IEQ
50 IEQ
50 IEQ
100 IEQ
100 IEQ
100 IEQ
150 IEQ
150 IEQ
150 IEQ
Group


8 Week Group Age at Diabetes (Red Bars are Recipients Prevented from Developing Diabetes)
Days When Became Diabetic
250
200
150
100
50
0
Controls
Controls
Controls
50 IEQ
50 IEQ
50 IEQ
50 IEQ
100 IEQ
100 IEQ
100 IEQ
150 IEQ
150 IEQ
150 IEQ
Group

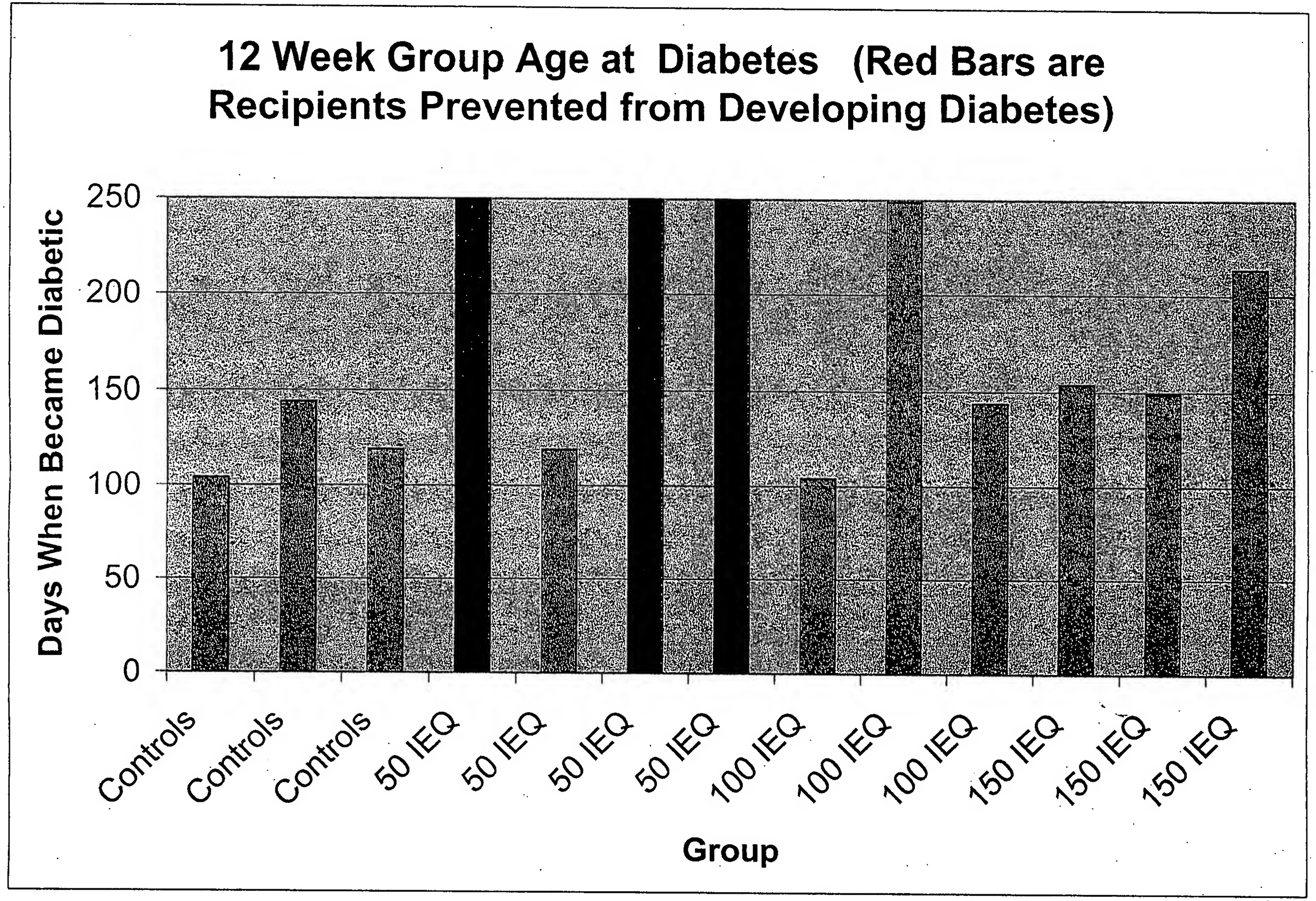

12 Week Group Age at Diabetes (Red Bars are Recipients Prevented from Developing Diabetes)
Days When Became Diabetic
250
200
150
100
50
0
Controls
Controls
Controls
50 IEQ
50 IEQ
50 IEQ
50 IEQ
100 IEQ
100 IEQ
100 IEQ
150 IEQ
150 IEQ
150 IEQ
Group

Exhibit A

CURRICULUM VITAE

David William Scharp, M.D.

Born: July 5, 1945
Washington, Illinois

Social Security Number: 327-36-7524

Marital Status: Wife: Jeanette

Children: Kevin Scharp
Daniel Scharp
Anna Scharp
David Bondurant
Melissa Bondurant

Pre-Medical Education: University of Missouri
Columbia, Missouri
1963-1966

Medical Education: Washington University School of Medicine
St. Louis, Missouri
1966-1970

Graduate Hospital Experience:

Intern in Surgery
7/1/70-6/30/71
Barnes Hospital/Washington University

Surgical Resident
7/1/71-6/30/72 & 7/1/74-6/30/76
Barnes Hospital/Washington University

Surgical Research Fellow
7/1/72-6/30/74
Washington University - Department of Surgery

Academic Positions:

Assistant Professor of Surgery
1976-1983
Washington University School of Medicine

Associate Professor of Surgery
1983-1991
Washington University School of Medicine

Professor of Surgery
1991-President
Washington University School of Medicine

Leave of Absence
1/1/94-7/1/95

Commercial Positions:

Novocell, Inc.
Chief Scientific Officer

Neocrin Company
Chief Scientific Officer 1/1/94-7/31/99
Executive Vice President of Medical Affairs 1/1/94-7/31/99
Executive Vice President for Research 1/1/94-11/1/95
Executive Vice President, Research and Development 11/1/95-President

McDonnell Douglas Corporation 1984-1987
Contractual Research Investigator
Electrophoretic Separation of Islet Cells

Cytotherapeutics, Inc. 1989-1993
Founding Scientist
Scientific Advisory Board Member
Contractual Research Investigator

Patents:

"Islet Isolation Process"
DE191613T – 1987
DE3650662D – 1998
EP0191613 – 1986
EP0191613 – 1989
EP0191613 – 1997
JP61183226 – 1986
US4868121 – 1989
US5322790 – 1994

"Method to Isolate Clusters of Cell Sub-Types from Organs"
AU1934988 – 1989
CA1340406 – 1999
EP0382727 – 1990
EP0382727 – 1991
JP2504222T – 1990
US5079160 – 1992
WO8809667 – 1988

"Implantable Biocompatible Immunoisolatory Vehicle for Delivery of Selected Therapeutic Products"
AT156344T – 1997
AU666118 – 1996
AU682796 – 1997
AU2004192 – 1992
AU3902095 – 1996
CA2109085 – 1992
CA2109085 – 2003
DE69221484D – 1997
DE69221484T – 1998
DK585368T – 1998
EP0585368 – 1994
EP0585368 – 1994
EP0585368 – 1997
ES2107537T – 1997
FI934545 – 1993
FI934545D – 1993
GR3025301T – 1998
HK1001832 – 1998
JP6507412T – 1994
NO308198B – 2000
NO933842 – 1993
SG47470 – 1998

US5798113 – 1998
US5800828 – 1998
US5871767 – 1999
US6083523 – 2000
US6322804 – 2001
US2002150603 – 2002
WO9219195 – 1992

"Methods for Coextruding Immunoisolatory Implantable Vehicles with a Biocompatible Jacket and a Biocompatible Matrix Core"
US5800829 – 1998

"Methods for Treating Diabetes by Delivering Insulin from Biocompatible Cell Containing Devices"

US5869077 – 1999

"Methods for Making Immunoisolatory Implantable Vehicles with a Biocompatible Jacket and a Biocompatible Matrix Core"
US5834001 – 1998
US5874099 –1999

"Use of Pouch for Implantation of Living Cells"
AU4788993 – 1994
CA2140905 – 1994
EP0655910 – 1995
EP0655910 – 1996
JP8500033T – 1996
US5916554 – 1999
WO9403154 – 1994

Hospital Appointments:

Assistant Surgeon 1976-1983
Associate Surgeon - 1983-Present
Barnes Hospital, St. Louis, Missouri

Attending Surgeon 1982-1985
Consulting Surgeon 1976-1982
Associate Chief of Surgery 12/86-9/90
Veterans Administration Medical Center, St. Louis, Missouri

Consulting Surgeon 1985-7/95
Acting Chief of Surgery 3/86-12/86
St. Louis Regional Hospital, St. Louis, Missouri

Consulting Surgeon 1976-1985
St. Louis Children's Hospital, St. Louis, Missouri

Consulting Surgeon 1976-1985
Chief of Surgery 1981-1982
St. Louis County Hospital, St. Louis, Missouri

Attending Surgeon 1976-1982
St. Louis City Hospital, St. Louis, Missouri

Licensure: Missouri 1970

Certification: American Board of Surgery 1979
Fellow American College of Surgery 1982
Recertification: American Board of Surgery 1989

Medical Societies:

American College of Surgeons
American Diabetes Association
American Federation of Clinical Research
American Pancreatic Association
American Society for Artificial Internal Organs
American Surgical Association
Association for Academic Surgery
Society of University Surgeons
Tissue Culture Association
American Society of Transplant Surgeons
The Cell Transplantation Society
United Network of Organ Sharing - Region 8
International Pancreas & Islet Transplantation Association

Honors and Awards:

St. Louis Surgical Society Award for Research
Recipient 1973 & 1974
NIH Research Career Development Award
Recipient 1977-1982
NIH NAIMMDD Site Visit Teams
Member 1980-1995

NIH Surgery, Anesthesiology, and Trauma Study Section
Member 1985-1989
Reserve Member Status 1989-1995
World Journal of Surgery
Guest Editor - Islet Transplantation Symposium 1984
"Separation of Islet Cells in Microgravity by Continuous-Flow Electrophoresis", NASA - McDonnell Douglas Astronautics Corp. - STS-8, Space Shuttle, "Challenger", Experiment 1984

Editorial Reviewer:
Diabetes, Surgery, Journal Clinical Investigation
Grant Reviewer
Canadian Diabetes Association
Medical Research Council of Canada
National Surgical Advisor - Digestive Disease Center of Excellence - The Humana Corporation 1986-1994
Alpha Omega Alpha - Washington University Chapter - Elected Faculty Member January 1988
Outstanding Profession/Scientific Employee - Federal Employee of the Year Award Program - St. Louis Federal Executive Board 1990
The Huddinge Hospital Transplant Lectureship - Annual Meeting of the Swedish Society for Medical Science, Stockholm, Sweden, December 1990
Council Member - Cell Transplantation Society 1992-Present
Council Member - International Pancreas & Islet Transplantation Association 1993-Present
Editorial Board
Cell Transplantation 1992-1993
Transplantation Science

Committee Appointments:
Washington University Animal Studies Committee
Chairman 1991-1994
Washington University Medical Center Alumni Association
Committee Member 1991-1994
International Juvenile Diabetes Research Foundation Medical Science Review Committee 1990-1993
UNOS Pancreas Subcommittee Member 1991-1995
American Society of Transplant Surgeons Program and Publications Committee 1989-1991
Academic Freedom and Tenure Hearing Committee
Member 1985-1991
Washington University Committee on the Humane Care of Laboratory Animal Member
Member 1984-1990

Operating Room Technician Program
Forest Park Community College
Advisory Committee 1976-1995
Chairman 1986-1995
Mid-America Transplant Association
Member Professional Advisory Board 1985-1995
American Cancer Society Institutional Research Grants
Washington University Committee for Cancer Research
Member 1979-1989
Chairman 1982-1989
"Health Views" - Editorial Advisory Board
Member 1984-1988
American Diabetes Association, St. Louis Chapter
Research Committee 1985-1988
Department of Surgery Animal Facility
Director 1980-1984
Washington University Faculty Senate
Member 1981-1983
Executive Committee of the Faculty Council
Member 1982-1984
Clinical Sciences Research Building Animal Surgery Suite
Director 1984-1985
Department of Engineering Master Degree Thesis Review Committee:
1979-John Bergen - "Kinetics of Insulin Secretion from Pancreatic Islets of Langerhans and Development of Islet Transplantation Chambers"
1980-Paul Aegerter - "Microencapsulation of Living Cells to Prevent Immunological Response"
1983-Shiow Meei Lin - "Testing of a Mathematical Model for Islet Transplantation Chambers"
1987-Donna Wilkinson - "Coating of Live Cells with Polysaccharide Derivatives"
1989-Mary Blanchard - "Quantification of Low Concentrations of Polysaccharide Derivatives and Their Effect on Cell Viability"
1990-Ph.D. Thesis Review, Donna Hawk-Reinhard - "Purification of Pancreatic Islets of Langerhans Using Cell Electrophoresis"
St. Louis VAMC Committees
Comprehensive Planning Committee
Chairman 1988-1990
Administrative Executive Board 1988-1990
Professional Standards Board 1988-1990
Research Committee 1988-1994
District Planning Board 1988-1990

Barnes Hospital Committees
Chaplaincy Committee 1992-1994
Emergency Room Committee 1978-1984
Search Committee for ER Director 1978-1984
Patient Education Parent Committee 1979
Surgery Patient Education Subcommittee Chairman 1981-1988
Tissue Culture Association
Publicity Chairman 1980

Invited Presentations, Selected:

The Kroc Foundation
Islet Transplantation Workshop 1974
Islet Transplantation Workshop 1979
Islet Transplantation Workshop 1982
National Institutes of Health
National Conference on Diabetes 1979
National Conference on Diabetes 1983
Juvenile Diabetes Foundation
Conference on Research Tissue 1981
National Meeting, Keynote Speaker 1984
International Scientific Research Conference 1985
German Diabetes Association, Giessen, West Germany
Islet Transplantation Workshop 1980
Islet Transplantation Workshop 1989
American Society of Artificial Organs
Annual Meeting - Keynote Speaker 1983
Session Co-Chairman 1987
International Symposium on Organ Transplantation in Diabetes
The Hague, Netherlands 1983
International Symposium on Kidney and Pancreas Transplantation
Perugia, Italy 1984
International Islet Transplantation Workshop
Canberra, Australia 1984
XII Congress of the International Diabetes Federation
Madrid, Spain 1985
XIII Congress of the International Diabetes Federation
Sydney, Australia 1989
National Disease Research Interchange
Human Tissue Conference 1985
Human Tissue Conference 1986
Human Tissue Conference 1987
Human Tissue Conference 1990

National Disease Research Interchange - Chairman of Task Force on "Biohazard and Contamination in the Use of Human Tissue and Organs"
Philadelphia, PA 1988
American Diabetes Association National Meeting - Session Co-Chairman for "Forms of Therapy" 1986
Visiting Scientist Program - University of Kansas Diabetes Center
Kansas City, Kansas 1986
Immunology of Diabetes Symposium - Member of International Advisory Committee
Edmonton, Canada 1986
International Symposium on Complications of Diabetes
The Hague, Netherlands 1986
Visiting Professorship - Department of Surgery - University of Minnesota
Minneapolis, Minnesota 1986
May 8th Endocrine Days
Victoria, British Columbia 1987
Second Annual Visiting Professorship in Diabetes - University of Wisconsin
Madison, Wisconsin 1987
First International Course on Transplantation
Venice, Italy 1987
Progress in Organ Transplants, Tissue Replacements and Implants
Sponsored by Biomedical Business International, New York 1987
Josiah Brown Memorial Symposium on Pancreas Beta Cell Transplantation
Los Angeles, California 1987
Seventh Workshop of the AIDSPIT Study Group
Igls, Austria 1988
First international Congress on Pancreatic and Islet Transplantation
Stockholm, Sweden 1987
Thirty-Fourth Annual Meeting of ASAIO, Invited Speaker "Modern Treatment of Insulin Dependent Diabetes"
Reno, Nevada 1988
Sixth Gordon Research Conference on Drug Carriers in Biology and Medicine
Plymouth, New Hampshire 1988
XII International Congress of the Transplantation Society
Sydney, Australia 1988
Second International Congress on Pancreatic and Islet Transplantation
Minneapolis, Minnesota 1989
Biology of Tissue Transplantation Symposium
Bethesda, Maryland 1989
Ninth Workshop of the AIDSPIT Study Group
Igls, Austria 1990
Society for Surgery of the Alimentary Tract Postgraduate Course, "Medical Aspects of Transplantation of the Liver, Pancreas and Intestine"
San Antonio, Texas 1990

Moderator for Pancreas Transplantation Scientific Session - American Society of Transplant Surgeons
Chicago 1990
UCLA Symposium on Molecular & Cellular Biology, "Tissue Engineering"
Keystone, Colorado 1990
The Huddinge Hospital Transplant Lectureship Annual Meeting of the Swedish Society for Medical Science
Stockholm, Sweden 1990
Third International Congress on Pancreatic and Islet Transplantation - Moderator and Plenary Speaker
Lyon, France 1991
European Association for the Study of Diabetes - Plenary Speaker
Dublin, Ireland 1991
Visiting Professor - University of Wisconsin
Madison, Wisconsin 1991
Moderator for Clinical Transplantation-Pancreas and Islets - XVIth International Congress of the Transplantation Society
Paris 1992
American Diabetes Association 53rd Annual Meeting - Plenary Speaker
Las Vegas, Nevada 1993
Fourth International Congress of Pancreas and Islet Transplantation - Plenary Speaker
Amsterdam 1993
IVth Joint Meeting of the Lawson Wilkins Pediatric Endocrine Society and the European Society for Pediatric Endocrinology - Symposium Speaker
San Francisco 1993
American Association for Clinical Chemistry
New York 1993

Publications

Abstracts:

1. Ballinger, W.F., Lacy, P.E., Scharp, D.W. Kemp, C.B., Knight, M. - Isografts and allografts of pancreatic islets in rats. Brit. J. Surg. 60:313, 1973

2. Kemp, C.B., Knight, M.J., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Islets of Langerhans injected into the portal vein of the diabetic rat. South African Journal of Surgery 11:135, 1973

3. Kemp, C.B., Knight, M.J., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Proceedings: Implantation of pancreatic islets into the portal vein of diabetic rats. Brit. J. Surg. 60:907, 1973

4. Scharp, D.W., Kemp, C.B., Knight, M.J., Murphy, J., Newton, W., Ballinger, W.F., Lacy, P.E. - Long term results of portal vein islet isografts and allografts in the treatment of Streptozotocin induced diabetes. Diabetes 23:359, 1974

5. Scharp, D.W., White, D.J., Ballinger, W.F., Lacy, P.E. - Transplantation of intact islets of Langerhans after tissue culture. In Vitro 9:364, 1974

6. Knight, M.J., Scharp, D.W., Kemp, C.B., Nunnelley, S.B., Ballinger, W.F., Lacy, P.E. - Cryopreservation of pancreatic islets. European Surgical Research 6(1):89, 1974

7. Ballinger, W.F., Murphy, J.J., Scharp, D.W., Hirshberg, G.E., Karl, R.C., Lacy, P.E. - Isolation and preservation of human islets of Langerhans for transplantation in the treatment of diabetes. European Society for Exp. Surg., Tenth Congress 1975

8. Griffith, R.C., Scharp, D.W., Ballinger, W.F., Lacy, P.E. - A morphologic study of intrahepatic portal vein islet isografts. Diabetes 34(2):419, 1975

9. Dodi, G., Scharp, D., Feldman, S., Maresca, B., Ballinger, W., Lacy, P. - Treatment of exocrine pancreatic dysfunction in diabetic rats by islet transplantation. European Surgical Research 9(1):98, 1977

10. Scharp, D.W., Merrell, R.C., Feldman, S., Ruwe, E., Feldmeier, M., Ballinger, W., Lacy, P. - Long term culture of islets of Langerhans utilizing a rotational culture method. In Vitro 13:174, 1977

11. Scharp, D., Krupin, T., Waltman, S., Oestrich, C., Feldman, S., Ballinger, W., Becker, B - Relationship of abnormal insulin release to fluorophotometry in experimental diabetes. Diabetes 27(2):435, 1978

12. Scharp, D.W., Merrell, R.C., Feldmeier, M.M., Downing, R., Ballinger; W.F. - Pseudo-islet formation and culture from canine isolated pancreatic cells. In Vitro 15:216, 1979

13. Rajotte, R.V., Scharp, D.W., Downing, R. Molnar, G.D., Ballinger, W.F. - The transplantation of frozen-thawed rate islets transported between centers. Diabetes 28:377, 1979

14. Downing, R. Scharp, D.W., Grieder, M., Ballinger, W.F. - Mass isolation of islets of Langerhans from the dog pancreas. Diabetes 28:426, 1979

15. Feldman, S.D., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Fetal pancreas isografts, cultured and uncultured to reverse Streptozotocin induced diabetes mellitus. The Association for Academic Surgery 12:116, 1979

16. Grieder, M.H., DeSchryver-Kecskemeti, K. Gingerich, R.L., Scharp, D.W. - In vitro studies using canine pseudo-islets and rat antrum cultures as models. UCLA Symposium, December 3, 1979

17. Scharp, D.W., Feldmeier, M.M., Rajotte, R.V., DeSchryver, K., Bell, M. - Human pseudo-islet formation, culture and preservation. Diabetes 29(2):18A, 1980

18. Gingerich, R.L., Scharp, D.W., Grieder, M.H., Dye, E.S., Mousel, K.A. - A new in vitro model to study secretion and biochemistry of pancreatic polypeptide (PP). Diabetes 29(2):30A, 1980

19. Bergen, J.F., Mason, N.S., Scharp, D.W., Sparks, R.E. - Insulin inhibition of islets in transplantation chambers. Presented at International Society for Artificial Organs Meetings, Paris, July 8-10, 1981

20. Sparks, R.E., Mason, N.S., Finley, T.C., Scharp, D.W. - Development, testing and modeling of an islet transplantation chamber, ASAIO Meetings, Chicago, April, 1982

21. Long, J.A., Adair, W.S., Scharp, D.W. - Hybridoma production against pancreatic cells. Diabetes 31(2):20A, 1982

22. Scharp, D.W., Hirshberg, G., Long, J.A. - The effect of islet dosage and time on rat portal vein isografts. Diabetes 31(2):162A, 1982

23. Scharp, D.W., Lacy, P.E. - The isolation and alteration of islet tissue for transplantation. The Tissue Culture Association Meeting, San Diego, June, 1982. In Vitro Suppl. 1, 1982

24. Long, J.A., Adair, W.S., Scharp, D.W. - An immunological approach to islet cell purification. J. Cell Biol. 95:4061, 1982

25. Sparks, R.E. Mason, N.S., Finley, T.C., Scharp, D.W. - Design of islet transplantation chambers giving a normal glucose tolerance test. ISAO Meetings, Kyoto, Japan, November, 1983

26. Sparks, R.E., Mason, N.E., Finley, T.C., Scharp, D.W. - Islet transplantation chamber models - assumption for insulin generation and glucose diffusion. International Symposium on Organ Transplantation in Diabetes, The Netherlands, September, 1983

27. Sparks, R.E., Mason, N.S., Scharp, D.W. - Some present directions in research on tissue transplantation chambers. International Conference on Artificial Organs, Glasgow, Scotland, September, 1983

28. Sparks, R.E., Mason, N.S., Finley, T.C., Scharp, D.W. - "A distributed source-model for hybrid artificial pancreas", presented by ASAIO, Toronto, Ontario, Canada, April, 1983

29. Scharp, D.W., Feldmeier, M.M., Olack, B.J., Swanson, C.J., O'Shaughnessey, S.F. - Electrophoretic purification of islet cells for transplantation. Diabetes 33(1):179A, 1984

30. Scharp, D.W., Rajotte, R.V., Kneteman, N.M., Lacy P.E. - Zero gravity electrophoresis of islet cells. 10th International Congress of the Transplantation Society Meeting, Minneapolis, August, 1984

31. Scharp, D.W., Lacy, P.E. - Human islet isolation and transplantation. Diabetes 34(1):5A, 1985

32. Kneteman, N.M., Alderson, D., Scharp, D.W. - Canine pancreatic islet allotransplantation: dose adjusted cyclosporine A vs azathioprine - steroid, Diabetes 34(1):62A, 1986

33. Alderson, D., Kneteman, N.M., Scharp, D.W. - The isolation of purified human islets of Langerhans. Diabetes 34(1):81A, 1986

34. Corlett, M.P., Fonseca, P., Scharp, D.W. - Detrimental effect of warm ischemia on islet isolation in rats and dogs with protection by oxygen free radical scavengers. Diabetes 36(1):223A, 1987

35. Scharp, D.W., Lacy, P.E., Finke, E.H., Olack, B.J. - Seven day culture of Ficoll purified human islets. Diabetes 36(1):222A, 1987

36. Corlett, M.P., Scharp, D.W. - Effect of warm ischemia on islet isolation in rats and dogs. The Association for Academic Surgery, Orlando, Florida, November 1-4, 1987

37. Misler, S., Gee, W., Scharp, D., Manchester, J., Falke, L. - Metabolically regulated potassium channels in human islet cells. Diabetes 37(1):6A, 1988

38. Atkinson, M.A., Maclaren, N.K., Riley, W.J., Scharp, D.W., and Holmes, L. - M_r 64,000 autoantibodies (64KA) predict insulin dependent diabetes (IDD). Diabetes 37(1):98A, 1988

39. Scharp, D.W., Lacy P.E., Ricordi, C., Boyle, P., Santiago, J.V., Gingerich, R.L., Anderson, C.B., Flye, M.W. - Human islet transplantation in patients with Type I diabetes. Presented at XII International Congress of the Transplantation Society, Sydney, Australia, August 14-19, 1988

40. Riley, W.J., Maclaren, N.K., Atkinson, M.A., Scharp, D.W., Lacy, P.E. - Islet autoantibodies in "prediabetes". Ninth International Workshop on the Immunology of Diabetes, Satellite Symposium on Beta Cell Destruction and Restoration in Type I Diabetes. November 27-29, Sydney, Australia, 1988

41. Atkinson, M.A., Maclaren, N.K., Riley, W.J., Scharp, D.W., Holmes, L. - The natural history and predictive value of M_r 64,000 islet cell antigen. Ninth International Workshop on the Immunology of Diabetes, Satellite Symposium of the International Diabetes Federation, Melbourne, Australia, 1988

42. Scharp, D.W., Chern, H.T., McCullough, C.S., Gingerich, R.L. - Lack of glucose induced insulin responsiveness in canine islets. 49th Annual Meeting of the American Diabetes Association, Detroit, Michigan, June 3-6, 1991. Diabetes 38(1):209A, 1989

43. Chern, H.T., Scharp, D.W., McCullough, C.S. - Cryogenic storage of purified canine islets. 49th Annual Meeting of the American Diabetes Association, Detroit, Michigan, June 3-6, 1989. Diabetes 38(1):246A, 1989

44. Callery, M.P., Ricordi, C., Scharp, D.W., Kamei, T., Lacy, P.E., Flye, M.W. - Hepatic insufficiency following portacaval shunting is prevented by prior intraportal pancreatic islet autotransplantation. The Society of University Surgeons, Baltimore, Maryland, 1989

45. Scharp, D.W., Corlett, M.P., Ricordi, C., Lacy, P.E. - Microbial contamination risk in human islet isolation. Study Group of the European Association for the study of Diabetes, Igls, Austria, 1989

46. Ricordi, C., Lacy, P.E., Socci, C., Finke, E.H. Dye, E., Olack, B.J., Swanson, C., Scharp, D.W. - Pancreatic islet isolation in mammals: Developments of the automated method. Study Group of the European Association for the Study of Diabetes, Igls, Austria, 1989

47. Callery, M.P., Kamei, T., Flye, M.W., Scharp, D.W. - Intrahepatic islet autografts in dogs function despite completely diverting portacaval shunt (PCS). Second International Congress on Pancreatic and Islet Transplantation, Minneapolis, Minnesota, 1989

48. McCullough, C., Chern, H.T., Scharp, D.W., Swanson, C.J., Olack, B.J., Davis, C.G. - Influence of transplant site on efficiency of canine islet cell auto-transplantation. Second International Congress on Pancreatic and Islet Transplantation, Minneapolis, Minnesota, 1989

49. Scharp, D.W. - islet Preservation - Short term for quality control testing. Presented at The Third International Conference on The Use of Human Cells, Tissues and Organs in Research. Washington, D.C., September 17-18, 1998

50. Falqui, L., Kowalski, L., Bittner, P., Olack, B.J., Scharp, D.W., Lacy, P.E. - Immunosuppressive regimens to prolong survival of islet xenografts transplanted across a wide species barrier (dog to mouse). Ninth Workshop of the AIDSPIT Study Group, Igls, Austria, January, 1990

51. Scharp, D.W., Lacy, P.E., Santiago, J.V., Boyle, P.J., Weide, L, Falqui, L., Marchetti, P, Gingerich, R.L., Cryer, P.E., Jaffe, A.S., McCullough, C.S., Anderson, C.B., Flye, M.W. - A clinical trial of islet transplantation. Ninth Workshop of the AIDSPIT Study Group, Igls, Austria, January, 1990

52. Marchetti, P., Falqui, L., Scharp, D.W., Lacy, P.E. - An improved method for large scale isolation of pure, viable islets of Langerhans from the adult porcine pancreas. Ninth Workshop of the AIDSPIT Study Group, Igls, Austria, January 1990

53. Scharp, D.W., Lacy, P.E., Santiago, J.V., McCullough, C.S., Weide, L.G., Falqui, L., Marchetti, P. Gingerich, R.L., Cryer, P.E., Jaffe, A.S., Anderson, C.B., Flye, M.W. - Preliminary results of clinical trials of human islet transplants. American Society of Transplant Surgeons 16th Annual Scientific Meeting, Chicago, Illinois, May 30-June 1, 1990

54. Marchetti, P., Scharp, D.W., Finke, E.H. - The isolation, function and xenotransplantation of porcine islets of Langerhans (PIL). 50th Annual Meeting of the American Diabetes Association, Atlanta, Georgia, June 16-19, 1990

55. Scharp, D.W., Lacy, P.E., McCullough, C.S., Weide, L., Boyle, P., Santiago, J.V., Gingerich, R., Cryer, P., Anderson, C.B., Flye, M.W. - Intraportal human islet transplants. XIII International Congress of The Transplantation Society, San Francisco, California, August 19-24, 1990

56. Finke, E.H., Marchetti, P., Swanson, C.J., Falqui, L., Lacy, P.E., Scharp, D.W. - Large scale isolation, function and transplantation of islets of Langerhans. XIII International Congress of the Transplantation Society, San Francisco, California, August 19-24, 1990

57. Olack, B.J., Swanson, C.J., McLear, M., Longwith, J., Scharp, D.W., Lacy, P.E. - Islet purification using Euroficoll gradients. XIII International Congress of the Transplantation Society, San Francisco, California, August 19-24, 1990

58. Scharp, D.W. - Clinical Trials. Presented at The Third International Conference on The Use of Human Cells, Tissues and Organs in Research. Washington, D.C., September 17-18, 1990

59. Swanson, C.J., Olack, B.J., Marchetti, P., Scharp, D.W. - Long-term metabolic and hormonal changes after intrasplenic canine islet transplantation. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

60. Olack, B.J., Marchetti, P., Swanson, C.J., Kneteman, N.M., Scharp, D.W. - No adverse effects of Cyclosporine A on either oral or intravenous glucose tolerance tests in Transplanted Dogs. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

61. McLear, M., Marchetti, P., Gingerich, R., Scharp, D.W. - The function of isolated human islets In Vitro. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

62. Marchetti, P., Swanson, C.J., Olack, B.J., McCullough, C.S., Scharp, D.W. - The effect of islet transplant site and mass on long-term metabolic function of canine islet autografts. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

63. Olack, B.J., Hill, A.C., Scharp, D.W., Lacy, P.E. - Automated purification of canine islets. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

64. McLear, M., Marchetti, P., Finke, E., Gerasimidi, A., Scharp, D.W., Lacy, P.E. - Porcine islet function: effects of arginine, leucine, and butyrate. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

65. Swanson, C.J., Marchetti, P., Newton, M., Scharp, D.W. - Effects of insulin therapy on glucose metabolism and insulin secretion in dogs and pancreatic islet autografts. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

66. Scharp, D.W., Lacy, P.E., Longwith, J.E. - Bacteriologic risks of human islet isolation for transplantation. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

67. Marchetti, P., Pfiffner, K., Scharp, D.W., Lacy, P.E., Gerasimidi, A., Finke, E.H. - Successful cryopreservation of porcine pancreatic islets (PI). Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

68. Marchetti, P., Swanson, C.J., Finke, E.H., Gerasimidi, A., Scharp, D.W., Lacy, P.E. - In Vitro function and xenotransplantation of long-term (3 weeks) cultured porcine islets of Langerhans. First international Congress on Xenotransplantation. Minneapolis, Minnesota, August 25-28, 1991

69. Marchetti, P., Giannarelli, Scharp, D.W., di Carlo, A., McLear, M., Lacy, P.E., Navalesi, R. - Metformin potentiates glucose stimulated insulin release: a study on diabetic patients and isolated human islets. European Association for the Study of Diabetes 27th Annual Meeting, Dublin, Ireland, September 10-14, 1991

70. Scharp, D.W., Marchetti, P., Swanson, C.J., Olack, B.J., Newton, M. - The effect of implantation site and islet mass on long-term survival and metabolic function of pure canine islet autografts. European Association for the Study of Diabetes 27th Annual Meeting, Dublin, Ireland, September 10-14, 1991

71. Marchetti, P., Finke, E., Pfiffner, K., Swanson, C., Gerasimidi-Vazeou, A., Olack, B., Scharp, D.W., Lacy, P.E. - Cryogenic storage of purified porcine islets. Presented at Study Group of the European Association for the Study of Diabetes, Igls, Austria, January 26-28, 1992

72. Scharp, D.W., Olack, B.J., Pfiffner, K., Gerasimidi-Vazeou, A., Finke, E.H., Lacy, P.E. - Preserving human islets prevents their rejection as xenografts. Presented at the First International Congress of the Cell Transplant Society, Pittsburgh, Pennsylvania, May 31-June 3, 1992

73. Marchetti, P., Scharp, D.W., Olack, B.J., Swanson, C.J., Bier, D., Cobelli, C., Toffolo, G., Newton, M., Navalesi, R. - Glucose metabolism, insulin sensitivity and glucagon secretion in dogs with intraportal or intrasplenic islet autografts. Presented at the First International Congress of the Cell Transplant Society, Pittsburgh, Pennsylvania, May 31-June 3, 1992

74. Marchetti, P., Scharp, D.W., Olack, B.J., Swanson, C.J. - Prediction of islet autograft failure by fasting glucagon:insulin ratio. Presented at the XIV International Congress of the Transplantation Society, Paris, France, August 16-21, 1992

75. Flavin, K.S., Scharp, D.W., Lacy, P.E. - Pre-islet transplant evaluation of psychiatric status using the National Institute of Mental Health (NIMH) Diagnostic Interview Schedule (DIS). Presented at the American Diabetes Association Meetings, San Antonio, Texas, June 20-23, 1992

76. Hawk-Reinhard, D.R., Scharp, D.W., Sparks, R.E. - Buffer solution temperature and velocity profiles in vertical micro-electrophoresis. Presented at American Institute of Chemical Engineers, New York, NY, November, 1992

77. Marchetti, P., Scharp, D.W., Finke, E., Pfiffner, K., Swanson, C., Gerasimidi-Vazeou, A., Olack, B., Navalesi, R., Lacy, P.E. - Insulin release upon perifusion and xenotransplantation of cryopreserved porcine islets. Presented at European Association for the Study of Diabetes, Igls, Austria, January 1992

78. di Carlo, A., Scharp, D.W., Gingerich, R.L., Ansara, M., Olack, B., Navalesi, R., Swanson, C. - Effect of culture and cryopreservation on insulin release from isolated, perifused human islets. Hormone and Metabolic Research 25:52, 1993

79. Marchetti, P., Scharp, D.W., Finke, E., Swanson, C.J., Gerasimidi-Vazeou, A., Olack, B.J., Navalesi, R., Lacy, P.E. - Prolongation of pig-to-mouse islet xenograft survival. Hormone and Metabolic Research 25:62, 1993

80. Hegre, O., Laurance, M., Harvey, J., Dionne, K., Appel, M., Scharp, D., Lacy, P.E. - Species variation in insulin secretory capacity of isolated islets. Hormone and Metabolic Research 25:58, 1993

81. Marchetti, P., Scharp, D.W., McLear, M., Finke, E.H., Olack, B.J., Swanson, C.J., Giannarelli, R. Navalesi, R., Lacy, P.E. - Pulsatile insulin secretion from isolated human pancreatic islets. Presented at the 53rd Annual Meeting of the American Diabetes Association Meeting, Las Vegas, Nevada, June, 1993

82. Shiroki, R., Poindexter, N., Mohanakumar, T., Scharp, D.W. - Rejection of human islet allografts in human lymphocyte reconstituted severe combined immunodeficient mice (Hu-Scid). Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

83. Marchetti, P., Scharp, D.W., Finke, E., Swanson, C.J., Gerasimidi-Vazeou, A., Olack, B.J., Navalesi, R., Lacy, P.E. - Effect of different temporary immunosuppressive therapies and low-temperature culture on pancreatic islet xenograft survival (pig-go-mouse). Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

84. Ansara, M.F., Saudek, F., Newton, M., Cryer, P.E., Scharp, D.W. - Islet transplantation prevents defective glucose counterregulation in diabetic dogs. Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

85. Scharp, D.W., Ansara, M.F., di Carlo, A., Flavin, K.S., White, N.H., Jendrisak, M.D., Lacy, P.E. - Intrasplenic islet transplants in kidney allograft recipients. Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

86. di Carlo, A., Scharp, D.W., Gingerich, R.L., Black, B.J., Swanson, C.J., Navalesi, R. - Insulin and glucagon release from isolated, perifused human islets following low temperature culture and cryopreservation. Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

87. Atkison, P., Zucker, P, Hramiak, I., Paul, T., McCallu, J., Stiller, KC.R., Scharp, D.W., Lacy, P.E., Olack, B., Finegood, D.T. - Continued insulin dependence despite normal insulin sensitivity and graft insulin secretion in a kidney/islet transplant patient. Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

88. Scharp, D.W., Swanson, C.J., Olack, B.J., Latta, P.P., Hegre, O.D., Gentile, F., Lacy, P.E. - Encapsulated human islet allografts in non-diabetic, Type 1 and Type 2 diabetic recipients. Presented at The 13th Workshop of the AIDSPIT Study Group, Igls, Austria, January 23-25, 1994. Horm. Metab. Res. 26:76, 1994

89. Scharp, D.W., Ansara, M.F., Brennan, D.C., White, N.H., Flavin, K.S., Zukosky, J.M., Cryer, P.E., Lacy, P.E. - Modification in methodology results in improvement in simultaneous kidney-islet success. Presented at the XVth World Congress of The Transplantation Society, Kyoto, Japan, August 28-September 2, 1994

90. Scharp, D.W., Brennan, D.C., White, N.H., Flavin, K.S., Lacy, P.E. - Increased islet mass raises rate of insulin independence in islet transplant recipients. Presented at IPITA 1995 Congress, Miami, Florida, June 18-22, 1995

Articles:

1. Merrick, A.W., Scharp, D.W. - Electroencephalography of resting behavior in cattle, with observations on the question of sleep, Am. J. Vet. Res., 32:1893-1897, 1971

2. Knight, M.J., Scharp, D.W., Kemp, C.B., Ballinger, W.F., Lacy, P.E. - Effects of cold storage on the function of isolated pancreatic islets, Cryobiology, 10:89-90, 1973

3. Kemp, C.B., Scharp, D.W., Knight, M.J., Ballinger, W.F., Lacy, P.E. - Importance of implantation site of pancreatic islet isografts in treatment of experimental diabetes, Surgical Forum 24:297-299, 1973

4. Kemp, C.B., Knight, M.J., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Transplantation of isolated pancreatic islets into the portal vein of diabetic rats, Nature 244:447, 1973

5. Scharp, D.W., Kemp, C.B., Knight, M.J., Ballinger, W.F., Lacy, P.E. - The use of Ficoll in the preparation of viable islets of Langerhans from the rat pancreas, Transplantation 16:686-689

6. Kemp, C.B., Knight, M.J., Scharp, D.W., Ballinger, W.F., Lacy, P.E. - Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats, Diabetologia 9:489-491, 1973

7. Scharp, D.W., Murphy, J.J., Newton, W.T., Ballinger, W.F., Lacy, P.E. - Transplantation of islets of Langerhans in diabetic Rhesus monkeys, Surgery 77:100-105, 1975

8. Scharp, D.W., Murphy, J.J., Newton, W.T., Ballinger, W.F., Lacy, P.E. - Application of an improved isolation technique for islet transplantation in primates and rats, Transplant. Proc. 6(1):739-741, 1975

9. Feldman, S., Hirshberg, G., Dodi, G., Raizman, M., Scharp, D., Ballinger, W., Lacy, P.E. - Intrasplenic islet isografts, Surgery 82:386-394, 1977

10. Karl, R.C., Scharp, D.W., Ballinger, W.F., Lacy, P.E. - Transplantation of insulin-secreting tissues, J British Society Gastroenterology - GUT 18:1062-1072, 1977

11. Griffith, R.C., Scharp, D.W., Hartman, B.K., Ballinger, W.F., Lacy, P.E. - A morphologic study of intrahepatic portal vein islet isografts, Diabetes 26:201-214, 1977

12. Feldman, S., Scharp, D., Hirshberg, G., Dodi, G., Ballinger, W., Lacy, P.E. - Streptozotocin-induced liver tumors, Transplantation 24:152-154, 1977

13. Feldman, S., Dodi, G., Haid, K., Scharp, D., Ballinger, W., Lacy, P.E. - Artificial hybrid pancreas, Surgical Forum 28:439-441, 1977

14. Scharp, D.W., Merrell, R.C., Feldman, S.D., Downing, R., Lacy P,E., Ballinger, W. - The use of gyrotational culture for the preservation of isolated islets, Surgical Forum 29:100-102, 1978

15. Krupin, T., Waltman, S.R., Scharp, D.W., Oestrich, G., Feldman, S.D., Becker, D., Ballinger, W.F., Lacy, P.E. - Ocular fluorophotometry in experimental diabetes mellitus: The effect of pancreatic islet isografts, Investigative Ophthalmology & Visual Science 18:1185-190, 1979

16. Downing, R., Scharp, D.W., Ballinger, W.F. - An improved technique for the isolation and identification of mammalian islets of Langerhans, Transplantation 29:79-83, 1980

17. Lacy, P.E., Davie, J.M., Finke, E.H., Scharp, D.W. - Prolongation of islet allograft survival, Transplantation 27:171-174, 1979

18. Scharp, D.W., Downing, R., Merrell, R.C., Grieder, M. - Isolating the elusive islet, Diabetes 29(1):19-30, 1980

19. Downing, R., Scharp, D.W., Merrell, R.C., Grieder, M., Ballinger, W.F. - Venous versus ductal distension of the dog pancreas for isolation of islets, Surgical Forum 30:422-424, 1979

20. Merrell, R.C., Scharp, D.W., Gingerich, R., Feldmeier, M.M., Grieder, M., Downing, R. - New approaches to the separation of islet cells, Surgical Forum 30:303-305, 1979

21. Feldman, S.D., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Fetal pancreas isografts, cultured and uncultured, to reverse streptozotocin induced diabetes mellitus, J. Surgical Research 29:309-318, 1980

22. Grieder, M.H., Gingerich, R.L., Scharp, D.W. - In Vitro studies of canine pseudo-islets. UCLA Symposium - Cellular Basis of Chemical Messengers in the Digestive System", Los Angeles, January 16-18, 1980

23. Silberberg, R., Scharp, D.W., Lesker, P.A. - Biochemical studies of bone from diabetic rats and rats bearing transplanted pancreatic islets, Experimental Cell Biology 49:61-67, 1981

24. Rajotte, R.V., Scharp, D.W., Downing, R., Preston, R., Molnar, G.D., Ballinger, W.F. - Pancreatic islet banking: the transplantation of frozen-thawed rat islets transported between Centers, Cryobiology 18:357-369, 1981

25. Britt, L.D., Scharp, D.W. - Formation of pseudo-islets from neonatal pig pancreas, Surgical Forum 31:354-356, 1980

26. Britt, L.D., Stojeba, P.C., Scharp, C.R., Grieder, M.H., Scharp, D.W. - Neonatal pig pseudo-islets: A product of selective aggregation, Diabetes 30:580-583, 1981

27. Britt, L.D., Scharp, D.W., Lacy, P.E., Slavin, S. - Transplantation of islet cells across major histocompatibility barriers after total lymphoid irradiation and infusion of allogeneic bone marrow cells, Diabetes 31:36-38, 1982

28. Bergen, J., Mason, N.S., Scharp, D.W., Sparks, R.E. - Insulin inhibition of islets in transplantation chambers, Artificial Organs 5:787-791, 1981

29. Paquette, T.L., Gingerich, R., Scharp, D.W. - Altered amidation of pancreatic polypeptide in culture, Biochemistry 20:7403-7408, 1981

30. Schmidt, R.E., Scharp, D.W. - Axonal dystrophy in experimental diabetic autonomic neuropathy, Diabetes 31:761-770, 1982

31. Yasunami, Y., Lacy, P.E., Scharp, D.W., Britt, L.D. - Isolation and transplantation of islet cells from the neonatal pig pancreas, Surgical Forum 33, 359-361, 1982

32. Gingerich, R.L., Scharp, D.W., Grieder, M.H., Dye, E.S., Mousel, K.A. - A new in vitro model for studies of pancreatic polypeptide secretion and biochemistry, Regulatory Peptides 5:13-25, 1982

33. Scharp, D.W., Long, J.A. - Studies of islet isolation and transplantation, Cryobiology 20:129-137, 1983

34. Long, J.A., Britt, L.D., Olack, B.J., Scharp, D.W. - Autotransplantation of isolated canine islets, Transplant Proc. 15:1332-1338, 1983

35. Schmidt, R.E., Santiago, B.P., Olack, B.J., Scharp, D.W. - The effect of pancreatic islet transplantation and insulin therapy on experimental diabetic autonomic neuropathy, Diabetes 32:532-540, 1983

36. Giddings, S.J., Rotwein, P., Chirgwin, J.M., Scharp, D.W., Permutt, M.A. - Analysis of insulin gene expression in human pancreas, Diabetes 32:777-780, 1983

37. Scharp, D.W. - Isolation and transplantation of islet tissue, World J. of Surgery 8:143-151, 1984

38. Scharp, D.W., Mason, N.S., Sparks, R.E. - Islet immunoisolation: the use of hybrid artificial organs to prevent islet tissue rejection, World J. of Surgery 8:221-229, 1984

39. Scharp, D.W. - Clinical feasibility of islet transplantation, Transplant Proc. 16(3):820-825, 1984

40. Heiken, J.P., Balfe, D.M., Picus, D., Scharp, D.W. - Radical pancreatectomy: Postoperative evaluation by CT, Radiology 153:211-215, 1984

41. Martin, K.W., Morian, J.P., Jr., Lee, J.K.T., Scharp, D.W. - Demonstration of splenic artery pseudoaneurysm by Magnetic Resonance Imaging, J. of Computer Assisted Tomography 9(1):190-192, 1985

42. Downing, R., Morrissey, S., Kiske, D., Scharp, D.W. - Does the purity of intraportal islet isografts affect their endocrine function?, J. Surg. Research 41(1):41-46, 1986

43. Scharp, D.W., Lacy, P.E., Alderson, D., Kneteman, N.M. - The procurement and distribution of cadaver human pancreas for islet isolation and transplantation. The Use of Human Tissues and Organs for Research and Transplant: Proceedings of the Second International Conference: National Diabetes Research Interchange April 1986:61-70

44. Scharp, D.W., Alderson, D., Kneteman, N.M. - Optimization of islet preparations for clinical transplantation. Transplant Proc. 18(6):1814-1816, 1986

59. Ricordi, C., Scharp, D.W., Lacy, P.E. - Mass isolation of human pancreatic islets for transplantation. Proceedings from XXVI World Congress of the International College of Surgeons, Milan, Italy, July 3-9, 1988, Monduzzi Editore S.p.A.

60. Corlett, M.P., Scharp, D.W. - The effect of pancreatic warm ischemia on islet isolation in rats and dogs, J. Surg. Research, 45:531-536, 1988

61. Ricordi, C., Kneteman, N.M., Scharp, D.W., Lacy, P.E. - Transplantation of cryopreserved human pancreatic islets into diabetic nude mice, World J. Surgery 12:861-865, 1988

62. Misler, S., Gee, W.M., Gillis, K.D., Scharp, D.W., Falke, L.C. - A metabolite-regulated ATP-sensitive K+ channel in human pancreatic islet cells, Diabetes 38:422-427, 1989

63. Scharp, D.W., Lacy, P.E., Ricordi, C., Boyle, P., Santiago, J., Cryer, P., Gingerich, R., Jaffe, A., Anderson, C.B., Flye, M.W. - Human islet transplantation in patients with Type 1 diabetes, Transplant Proc 21 (No. 1):2744-2745, February 1989

64. Scharp, D.W. - Islet Transplantation, Diabetes 38: (Suppl. 1):323-325, 1989

65. Ricordi, C., Lacy, P.E., Scharp, D.W. - Automated islet isolation from human pancreas, Diabetes 38 (Suppl. 1):140-142, 1989

66. Kneteman, N.M., Alderson, D., Scharp, D.W., Lacy, P.E. - Prolonged cyropreservation of purified human pancreatic islets, Diabetes 38:176-178, 1989

67. Kneteman, N.M., Alderson, D., Scharp, D.W., Lacy, P.E. - Long-term cryogenic storage of purified adult human islets of Langerhans, Diabetes 38:386-396, 1989

68. Brelje, T.C., Scharp, D.W., Sorenson, R.L. - Three-dimensional imaging of intact, isolated islets of Langerhans using Confocal microscopy, Diabetes 38:808-814, 1989

69. Schmidt, R.E., Plurad, S.B., Olack, B.J., Scharp, D.W. - The effect of pancreatic islet transplantation and insulin therapy on neuroaxonal dystrophy in sympathetic autonomic ganglia of chronic Streptozotocin diabetic rats, Brian Research 497:393-398, 1989

70. Callery, M.P., Ricordi, C., Scharp, D.W., Kamei, T., Swanson, C.J., Lacy, P.E., Flye, M.W. - Hepatic insufficiency following portacaval shunting is prevented by prior intraportal pancreatic islet autotransplantation. Surgery 106:257-266, 1989

71. Dotta, F., Colman, P.G., Nayak, R.C., Lombardi, D., Scharp, D.W., Andreani, D., Pontieri, G.M., di Mario, U., Lenti, L., Eisenbarth, G.S. - Ganglioside expression in human pancreatic islets, Diabetes 38:1478-1483, 1989

72. Permutt, M.A., Koranyi, L., Keller, K., Lacy, P.E., Scharp, D.W., Mueckler, M. - Cloning and functional expression of a human pancreatic islet glucose-transporter cDNA, Proc Natl Acad Sci USA 86:8688-8692, 1989

73. Callery, M.P., Kamei, T., Flye, M.W., Davis, C.G., Swanson, C.J., Olack, B.J., Lacy, P.E., Scharp, D.W. - Intrahepatic islet autografts in dogs function despite completely diverting portacaval shunt, Transplant Proc 22:767-770, 190

74. Scharp, D.W., Lacy, P.E., Santiago, J.V., McCullough, C.S., Weide, L.G., Falqui, L., Marchetti, P., Gingerich, R.L., Jaffe, A.S., Cryer, P.E., Anderson, C.B., Flye, M.W. - Insulin independence after islet transplantation into Type 1 diabetic patient, Diabetes 39:515-518, 1990

75. Atkinson, M.A., Maclaren, N.K., Riley, W.J., Scharp, D.W., Lacy, P.E. - 64,000 M_r autoantibodies as predictors of insulin dependent diabetes, Lancet 335:1357-1360, 1990

76. Scharp, D.W., Olack, B.J., Swanson, C.J., Marchetti, P. - Normal reproductive function in dogs with islet autograft, Transplant Proc 22(5):2403-2404, 1990

77. Chern, H.T., Scharp, D.W. - Viability assessment of cryopreserved canine islets in vivo, Endocrine Surgery 7(3):355-360, 1990

78. Medoff, G., Sagal, S., Krogstad, D., Peters, M., Hogan, J., Scharp, D. - Clinical Pathology Conference - Abdominal pain, fever, and weight loss in a 61 year-old Arabian Man, Am J Medicine 89:83-90, 1990

79. Ricordi, C., Gray, D.W.R., Hering, B.J., et al - Islet isolation assessment in man and large animals, Acta Diabetoal. Lat. 27:185-195, 1990

80. Atkinson, M.A., Maclaren, N.K., Holmes, L., Scharp, D.W., Lacy, P.E., Riley, W.J. - The natural history and predictive value of M_r 64,000 autoantibodies for insulin-dependent diabetes mellitus, J Autoimmunity 3:41-45, 1990

81. Atkinson, M.A., Holmes, L.H., Scharp, D.W., Lacy, P.E., Maclaren, N.K. - No evidence for serological autoimmunity to islet cell heat shock proteins in insulin dependent diabetes, J Clin Invest 87:721-724, 1991

82. Scharp, D.W., Lacy, P.E., Santiago, J.V., McCullough, C.S., Weide, L.G., Boyle, P.J., Falqui, L., Marchetti, P., Ricordi, C., Gingerich, R.L., Jaffe, A.S., Cryer, P.E., Hanto, D.W., Anderson, C.B., Flye, M.W., - Results of our first nine intraportal islet allografts in Type 1, insulin-dependent diabetic patients, Transplantation 51:76-85, 1991

83. Finke, E., Marchetti, P., Falqui, L., Swanson, C., McLear, M., Olack, B., Scharp, D.W., Lacy, P.E. - Large scale isolation, function and transplantation of islets of Langerhans from the adult pig pancreas, Transplant Proc 23(1):772-773, 1991

84. Olack, B., Swanson, C., McLear, M., Longwith, J., Scharp, D.W., Lacy, P.E. - Islet purification using Euroficoll gradients, Transplant Proc 23(1):774-776, 1991

85. Scharp, D.W., Lacy, P.E., Weide, L.G., Marchetti, P., McCullough, C.S., Flavin, K., Santiago, J.V. - Intraportal islet allografts: the use of a simulation index to represent functional results, Transplant Proc 23(1):796-798, 1991

86. Falqui, L., Finke, E.H., Carel, J.C., Scharp, D.W., Lacy, P.E. - Marked prolongation of human islet xenograft survival (human to mouse) by low-temperature culture and temporary immunosuppression with human and mouse anti-lymphocyte sera, Transplantation 51:1322-1324, 1991

87. Scharp, D.W., Lacy P.E. - The clinical feasibility of human islet transplantation, Clinical Diabetes 42-45, May/June, 1991

88. Marchetti, P., Finke, E.H., Gerasimidi-Vazeou, A., Falqui, L., Scharp, D.W., Lacy P.E.- Automated large scale isolation, *in vitro* function and xenotransplantation of porcine islets of Langerhans, Transplantation 52:209-213, 1991

89. Kowalski, L., Falqui, L., Lacy, P.E., Scharp, D.W. - Production of marked prolongation of survival of canine islet xenografts in mice by antilymphocyte sera and L3T4 antibody, Transplantation 52:1094-1097, 1991

90. Marchetti, P., Swanson, C., McLear, M., Finke, E.H., Gerasimidi-Vazeou, A., Scharp, D.W., Lacy, P.E. - In vitro function and xenotransplantation of long-term (3 weeks) cultured porcine islets of Langerhans, Transplant Proc 24(2):637, 1992

91. Kneteman, N.M., Marchetti, P., Tordjman, K., Bier, D.M., Santiago, J.V., Swanson, C.J., Olack, B.J., Scharp, D.W. - Effect of cyclosporine on insulin secretion and insulin sensitivity in dogs with intrasplenic islet autotransplants, Surgery 111(4):430-437, 1992

92. Scharp, D.W., Marchetti, P., Swanson, C.J., Newton, M., McCullough, C.S., Olack, B.J. - The effect of transplantation site and islet mass on long-term survival and metabolic and hormonal function of canine purified islet autografts, Cell Transplantation 1:245-254, 1992

93. Misler, S. Barnett, D.W., Pressel, D.M., Gillis, K.D., Scharp, D.W., Falke, L.C. - Stimulus-secretion coupling in B cells of transplantable human islets of Langerhans: evidence for a critical role for Ca^{2+} entry, Diabetes 41:662-670, 1992

94. Marchetti, P., Scharp, D.W., Longwith, J.E., Swanson, C.J., Olack, B.J., Gerasimidi-Vazeou, A., Finke, E.H., Lacy, P.E. - Prevention of contamination of isolated porcine islets of Langerhans, Transplantation 53(6):1364-1366, 1992

95. Olack, B.J., Hill, A., Scharp, D.W., Lacy, P.E. - Automated purification of canine islets, Transplant Proc 24(3):1003-1004, 1992

96. Marchetti, P., Pfiffner, K., Scharp, D.W., Lacy, P.E., Gerasimidi-Vazeou, A., Finke, E.H. - Successful cryopreservation of porcine pancreatic islets, Transplant Proc 24(3):1005-1006, 1992

97. Scharp, D.W., Lacy, P.E., McLear, M., Longwith, J.E., Olack, B.J. - The bioburden of 590 consecutive human pancreata for islet transplant research, Transplant Proc 24(3):974-975, 1992

98. McLear, M., Marchetti, P., Finke, E., Gerasimidi-Vazeou, A., Scharp, D.W., Lacy, P.E. - Porcine islet function: Effects of arginine, Leucine, and Butyrate, Transplant Proc 24(3):980-982, 1992

99. Marchetti, P., Finke, E.H., Swanson, C., Gerasimidi-Vazeou, A., Olack, B., Scharp, D.W., Navalesi, R., Lacy, P.E. - The potential of porcine islet xenotransplantation in the therapy of diabetes, Diab. Nutr. Metab. 5 (Suppl 1):151-154, 1992

100. Marchetti, P., Scharp, D.W., Olack, B.J., Swanson, C.J., Bier, D., Colelli, C., Toffolo, G., Newton, M., Navalesi, R. - Glucose metabolism, insulin sensitivity and glucagon secretion in dogs with intraportal or intrasplenic islet autografts, Transplant Proc 24(6):2828-2829, 1992

101. Marchetti, P., Olack, B., Swanson, C., Newton, M., Scharp, D.W. - Assessment of long-term (1 year) graft survival and metabolic hormonal changes after intrasplenic canine pancreatic microfragment transplantation, Diabetes & Metabolism 19:17-24, 1993

102. Barnett, D.W., Pressel, D.M., Chern, H.T., Scharp, D.W., Misler, S. - cAMP-Enhancing agents "permit" stimulus-secretion coupling in canine pancreatic islet ß-cells, J. Membrane Biol 138:113-120, 1994

103. Ansara, M.F., Saudek, F., Newton, M., Cryer, P.E., Scharp, D.W. - Pancreatic islet transplantation prevents defective glucose counter-regulation in diabetic dogs, Transplant Proc. 26(2):664-665, 1994

104. Shiroki, R., Poindexter, N., Mohanakumar, T., Scharp, D.W. - Rejection of human islet allografts in human lymphocyte-reconstituted severe combined immunodeficient mice, Transplant Proc. 26(2):716-717, 1994

105. Marchetti, P., Scharp, D.W., Finke, E., Swanson, C.J., Gerasimidi-Vazeou, A., Olack, B.J., Navalesi, R., Lacy, P.E. - Effect of different temporary immunosuppressive therapies and low-temperature culture on pancreatic islet xenograft survival (Pig-to-Mouse), Transplant Proc. 26(2):760-761, 1994

106. di Carlo, A., Scharp, D.W., Gingerich, R.L., Giannarelli, R., Ansara, M., Olack, B.J., Swanson, C.J., Navalesi, R. - Insulin and glucagon release from isolated, perifused human islets following low-temperature culture and cryopreservation, Transplant Proc. 26(2):821-822, 1994

107. Marchetti, P., Scharp, D.W., Pfiffner, K., Swanson, C.J., Finke, E.H., Olack, B.J., Gerasimidi-Vazeou, A., McLear, M., Lacy, P.E. - Cryogenic storage of isolated, purified porcine pancreatic islets, Transplantation 57:340-346, 1994

108. Marchetti, P., Kneteman, N.M., Swanson, C.J., Olack, B.J., Scharp, D.W. - Effect of cyclosporin treatment on metabolic and hormonal responses to mixed meal plus oral glucose in dogs with intrasplenic pancreatic islet autograft, Experimental Medicine, In Press

109. Marchetti, P., Scharp, D.W., McLear, M., Gingerich, R., Finke, E.H., Olack, B.J., Swanson, C.J., Giannarelli, R., Navalesi, R., Lacy, P.E. - Pulsatile insulin secretion from isolated human pancreatic islets, Diabetes 43:827-830, 1994

110. Shiroki, R., Poindexter, N.J., Woodle, E.S., Hussain, M.S., Mohanakumar, T., Scharp, D.W. - Human peripheral blood lymphocyte reconstituted severe combined immunodeficient (hu-PBL-SCID) mice: a model for human islet allograft rejection, Transplantation 58:1555-1562, 1994

111. Scharp, D.W., Swanson, C.J., Olack, B.J., Latta, P.P., Hegre, O.D., Doherty, E.J., Gentile, F.T., Flavin, K.S., Ansara, M.F., Lacy, P.E. - Protection of encapsulated human islets implanted without immunosuppression in patients with Type I and Type II diabetes and in non-diabetic controls, Diabetes 43:1167, 1994

112. Marchetti, P., Scharp, D.W., Finke, E.H., Giannarelli, R., Swanson, C.J., Olack, B.J., Lacy, P.E., Navalesi, R. - Factors affecting in vitro and in vivo function of isolated porcine islets, Transplant Proc. 26(6):3419-3420, 1994

113. di Carlo, A., Scharp, D.W., Gingerich, R.L., Ansara, M.F., Olack, B.J., Swanson, C.J., Giannarelli, R., Navalesi, R. - C-peptide secretion from isolated perifused human islets: effect of culture and cryopreservation, Transplant Proc. 26(6) 3399, 1994

114. Perdrizet, G.A., Rewinski, M.J., Schweizer, R.T., Scharp, D.W. - Heat shock and recovery protects pancreatic islets from warm ischemic injury, Transplant Proc. 26(6) 3477-3478, 1994

115. Marchetti, P., Scharp, D.W., Finke, E., Swanson, C.J., Gerasimidi-Vazeou, A., Olack, B.J., Navalesi, R., Lacy, P.E. - Anti-CD4 antibody treatment in xenografts of differently immunomodulated porcine islets, Transplant Proc. 26(3):1132, 1994

116. Brennan, D.C., Scharp, D.W., White, N.H., Flavin, K.S., Zukosky, J.M., Ansara, M.F., Cryer, P.E., Lacy, P.E. - Modification of methodology results in improvements in simultaneous kidney-islet success, Transplant Proc. 27(1):1349-1350, 1995

117. Chern, H.T., Scharp, D.W. - Successful long-term cryopreservation of highly purified canine islets, European Surg Res 27:167-175, 1995

118. Marchetti, P., Scharp, D.W., Finke, E.H., Swanson, C.J., Olack, B.J., Gerasimidi-Vazeou, A., Giannarelli, R., Navalesi, R., Lacy, P.E. - Prolonged survival of discordant porcine islet xenografts, Transplantation 61:1100-1102, 1996

119. Hill, R.S., Cruise, G.M., Hager, S.R., Lamberti, F.V., Yu, X., Garufis, C.L., Yu, Y., Mundwiler, K.E., Cole, J.F., Hubbell, J.A., Hegre, O.D., Scharp, D.W. - Immunoisolation of adult porcine islets for the treatment of diabetes mellitus, Annals of the NY Academy of Sciences 831:332-343, 1997

120. Cruise, G.M., Hegre, O.D., Scharp, D,W., Hubbell J.A., - A sensitivity study of the key parameters in the interfacial photopolymerization of poly(ethylene glycol) diacrylate upon porcine islets, Biotech. Bioeng. 57:655-665, 1998

121. Cruise, G.M., Scharp, D.W., Hubbell, J.A. - Characterization of permeability and network structure of interfacially photopolymerized poly(ethylene glycol) diacrylate hydrogels, Biomaterials 19:1287-1294, 1998

122. Cruise, G.M., Hegre, O.D., Lamberti, F.V., Hager, S.R., Hill, R., Scharp, D.W., Hubbell, J.A. - *In vitro* and *in vivo* performance of porcine islets encapsulated in interfacially photopolymerized poly(ethylene glycol) diacrylate membranes, Cell Transplantation, 8:293-306, 1999

Letters, Reviews and Chapters:

1. Karl, R., Scharp, D.W., Ballinger, W.F., Lacy, P.E. - Progress Report: transplantation of insulin secreting tissue, Gut. 18:1062-1072, 1977

2. Lacy, P.E., Scharp, D.W., Ballinger, W.F. - Transplantation of the islets of Langerhans - future directions. 14th International Congress of Therapeutics, p. 105, L'Expansion Scientique Francaise, Montpellier, France, 1977

3. Scharp, D.W. - Rejection of islet "isografts" in a strain of Lewis rats, Transplantation, 31:229-230, 1981

4. Rajotte, R.V., Scharp, D.W., Molnar, G.D., Preston, R. - Feasibility of low temperature banking for pancreatic islet cell transplantation. In "Islet Isolation, Culture, and Cryopreservation", Ed. K. Federlin and R.G. Bretzel. Thieme-Stratton, Inc., p. 124-135, 1981

5. Scharp, D.W., Downing, R., Merrell, R.C., Nunnelly, S., Kiske, D., Grieder, M.H. - New approaches in the methods for isolating mammalian islets of Langerhans utilizing the dog pancreas. In "Islet Isolation, Culture, and Cryopreservation". Ed. K. Federlin and R.G. Bretzel. Thieme-Stratton, Inc. p. 9-31, 1981

6. Slavin, S., Britt, L.D., Scharp, D.W., Lacy, P.E. - Treatment of experimental diabetes with histoincompatible islet allografts in rats conditioned with total lymphoid irradiation. In "Diabetic Renal-Retinal Syndrome", Volume 2. Ed. E.A. Friedman and E.A. L'Experance. Grune and Stratton, Inc., NY, p. 519-528, 1982

7. Chen, Y., Mason, N.S., Scharp, D.W., Ballinger, W.F., Sparks, R.E. - Collagenase immobilized on cellulose acetate membranes. In "Biomaterials: Interfacial Phenomena and Applications". Ed. S.L. Cooper and N.A. Peppas, American Chemical Society, Washington, D.C., p. 483-491, 1982

8. Scharp, D.W. - Hybrid Artificial Pancreas Panel Discussion. Transactions of the American Society for Artificial Internal Organs, 28:642-643, 1982

9. Scharp, D.W. - Clinical Feasibility of Islet Transplantation, International Symposium on Organ Transplantation in Diabetes, The Netherlands 1983, Transplant Proc. 16:820-825, 1984

10. Scharp, D.W., Mason, N.S., Sparks, R.E. - The use of hybrid artificial organs to provide immunoisolation to endocrine grafts. In "Bone Marrow & Organ Transplantation", Ed. S. Slavin, Elsevier Science Publishers, New York, p. 601-618, 1984

11. Scharp, D.W., Long, J.A., Feldmeier, M.M., Olack, B.J., O'Shaughnessy, S., Swanson, C.J. - Automated methods for isolating and purifying islet cells from the pancreas. In "Methods in Diabetes Research", Ed. Larner and Pohl, New York, John Wiley & Sons, p. 237-243, 1984

12. Scharp, D.W., Long, J.A., Feldmeier, M.M., Olack, B.J., O'Shaughnessy, S., Swanson, C.J. - Automated methods of mass islet isolation. In "Methods in Diabetes Research", Ed. Larner and Pohl, New York, John Wiley & Sons, p. 225-236, 1984

13. Slavin, S., Weiss, L., Weigensberg, M., Morecki, S., Fuks, Z., Britt, L., Lacy, P.E., Scharp, D.W. - Tollerance to alloantigens in rodents and reversal of streptozotocin-induced diabetes in tolerant rats by histo-incompatible islet allografts. In "Bone Marrow & Organ Transplantation", Ed. S. Slavin, Elsevier Science Publications, New York, 1984

14. Williamson, J.R., Olack, B.., Rowald, E.A., Chang, K., Scharp, D.W. - Effects of insulin treatment on collagen crosslinking in streptozotocin diabetic rats. In "Lessons from Animal Diabetes", Eds. E. Shafrir and A. Renold, John Libbey, London, 1984, p. 402-404

15. Lacy, P.E., Scharp, D.W. - Islet Transplantation in treating diabetes, Ann. Rev. Med,. 37:33-40, 1986

16. Scharp, D.W. - The elusive human islet: Variables involved in its effective recovery. In "Transplantation of the Endocrine Pancreas in Diabetes Mellitus", Eds. R. van Schifgaarde and M.A. Hardy, Elsevier Science Publications, Amsterdam, 1988, p. 97-107

17. Scharp, D.W., Lacy, P.E. - Islet transplantation: a review of the objective, the concepts, the problems, the progress and the future. In "Transplantation of the pancreas", Eds. J.M. Dubernard and D.E.R. Sutherland, Kluwer Academic Publishers, The Netherlands, 1989, p. 455-478

18. Scharp, D.W., Lacy, P.E. - Clinical islet transplantation. In "Excerpta Medica International Congress Series", Eds. R. Larkins, P. Zimmet, D. Chisholm, Elsevier Science Publishers, Amsterdam, 1989, p. 721-724

19. McCullough, C.S., Scharp, D.W., Lacy, P.E. - Human islet isolation and transplantation. In "Immunologische and Dyamische Aspekte der Insulinsubstitution beim Diabetes Mellitus", Ed. R.G. Bretzel, Springer-Verlag, Heidelberg, 1990, p. 260-267

20. Atkinson, M.A., Maclaren, N.K., Scharp, D.W. - No role for 65KD heatshock protein in diabetes, (Letter) Lancet 336:1250-1251, 1990

21. Lacy, P.E., Scharp, D.W. - Islet cell transplantation. In "Diabetes Mellitus", Fourth Edition, Eds. H. Rifkin and D. Porte, Jr., Elsevier Science Publications, New York, 1990, P. 880-886

22. McCullough, C.S., Scharp, D.W. - Pancreas and islet transplantation for the treatment of Type I diabetes mellitus. In "Surgical Management of the Diabetic Patient", Eds. M. Bergman and G.A. Sicard, Raven Press, Ltd., New York, 1991, p. 349-362

23. Scharp, D.W. - Islet Transplantation: Approaches to a Clinical Therapy. In "Perspectives in General Surgery", Vol 3, No. 1, Ed. Barry A. Levine, Quality Medical Publishers, St. Louis, MO, 1992, p. 93-105

24. Scharp, D.W. - Islet quality control testing and the islet isolation laboratory. In "Pancreatic Islet Cell Transplantation", Ed. Camillo Ricordi, R.G. Landes Company, Austin, 1992, p. 82-88

25. Scharp, D.W. - Clinical islet transplantation - registry report, accomplishments in the past and future research needs, Invited Commentary, Cell Transplantation 2:299, 1993

26. Scharp, D.W. - Pancreatic Islet Transplantation. In "Atlas of Organ Transplantation, Ed. M. Wayne Flye, W.B. Saunders Company, Philadelphia, PA, 1994, p. 209-220

27. Ansara, M.F., Cryer, P.E., Scharp, D.W. - Perioperative Management of the Diabetic Patient. In "Care of the Surgical Patient", Ed. A. McHugh, Scientific American Medicine, New York, NY, In Press

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ART UNIT	PAPER NUMBER
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DATE MAILED: 12/16/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/660,924	LATTA, PAUL P.	
	Examiner	Art Unit	
	Michail A Belyavskyi	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *12 October 2004*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *2-9* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *2-9* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

1. The **examiner** of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Michail Belyavskyi, Group Art Unit 1644, Technology Center 1600

Claims 2-9 are pending

2. Applicant's election of primary cell species ,allogenic cells species and without immunosuppression in the reply filed on 10/12/04 is acknowledged. Because applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.03(a)).

Claims 2-9 are under consideration in the instant application.

3. The title of the invention is not descriptive. A new title is required that is clearly indicative of the invention *to which the claims are directed.*

4. Applicant notes that an IDS was submitted with the prior application 09/226742. However these citations have been crossed out as said references cited in said parent application cannot be found. Applicant is invited to resubmit such references to complete the instant file. The examiner apologizes for any inconvenience to applicant for having to resubmit such documents.

5. The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

6. Claims 2-9 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. **This is a New Matter rejection.**

" A method of preventing onset of Type I diabetes in a mammal" claimed in claim 2 represent a departure from the specification and the claims as originally filed. The passages pointed by the applicant do not provide a clear support for the "A method of preventing onset of Type I diabetes in a mammal". The specification and the claims as originally field only support " A method of preventing diseases".

7. Also an issue that Claims 2-9 are rejected under 35 U.S.C. 112, first paragraph, because the specification, does not reasonably provide **enablement** for a method of preventing onset of Type I diabetes in a mammal , comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane.

The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and or use the invention commensurate in scope with this claim.

The specification disclosure does not enable one skilled in the art to practice the invention without an undue amount of experimentation.

Factors to be considered in determining whether undue experimentation is required to practice the claimed invention are summarized *In re Wands* (858 F2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988)). The factors most relevant to this rejection are the scope of the claim, the amount of direction or guidance provided, the limited working examples, the unpredictability in the art and the amount of experimentation required to enable one of skill in the art to practice the claimed invention.

The specification only discloses the effects of the implanting of insulin-producing cells on the level of blood glucose using streptozotocin-induced diabeties in murine experimental model. (See Examples 1-2 in particular). Examples 3-7 in the instant Specification are prophetic examples that indicate what the inventor thinks might happen in the experiments which have not actually been performed. The specification does not adequately teach how to effectively prevent onset of type I diabetes in mammal predisposed to type I diabetes , comprising implanting insulin-producing cells encapsulated in a biologically-compatible membrane. Mestas et al (J. of Immunology, 2004, 172, pages 2731-238) teach that there exist significant differences between mice and humans in immune system development, activating and response to challenge in both the innate and adaptive arms. As therapies for human diseases become ever more sophisticated and specifically targeted it becomes increasing important to understand the potential limitations

of extrapolating data from mice to humans. The literature is littered with the examples of therapies that work well in mice but fail to provide similar efficacy in humans. Teuveson et al., (Immun. Review 1993, N136, pages 101-107) teach that one problem with rodent models of transplantation is that rejection is easily overcome in said models in comparison to the difficulty of overcoming allograft rejection in human (see page 100 in particular). Teuveson et al., further teach that " however today's small animal models seem to be insufficient to produce data for clinical decision-making" and further raises doubt as to whether large animal models can be applied to clinical situations, due to species-specific reactions to treatment (see page 101 in particular). Feldman et al (Transplant. Proc. 1998, 30, 4126-4127) teach that "while it is not difficult to study the pathogenesis of animal models of disease, there are multiple constraints on analyses of the pathogenesis of human disease, leading to interesting dilemmas such as how much can we rely on and extrapolate from animal models in disease". In addition, Cochlovius et al (Modern Drug Discovery, 2003, pages 33-38) teach that in contrast to in vitro models, and partly animal-human xenograft systems, tissue cells in vivo seems to express molecules for defense against cellular immune systems as well as against complement. Although these defense mechanisms are still poorly understood, they provide some hints as to why many potential therapeutics perform marvelously in vitro but a fairly high portion of them still fail *in vivo*. Moreover, since the method of treating and preventing onset of type I diabetes in mammal predisposed to type I diabetes , comprising implanting insulin-producing cells encapsulated in a biologically-compatible membrane can be species- and model-dependent (see Van Noort et al. International Review of Cytology, 1998, v.178, pages 127-204, Table III in particular) , it is not clear that reliance on the *in vivo* murine data accurately reflects the relative any mammal and human efficacy of the claimed therapeutic strategy. Van Noort et al., further indicate factors that effect immune response such as genetic, environmental and hormonal (Page 176, Paragraph 3). The ability of a host to enhance an immune response will vary depending upon factors such as the condition of the host and burden of disease.

Thus, as has been discussed supra, the state of the art is that it is unpredictable form the *in vivo* murine data disclosed in the specification as whether the instant invention can be used for the *in vivo* treatment of diabetes in any mammals including human. Therefore, it is not clear that the skilled artisan could predict the efficacy of a method of preventing onset of type I diabetes in mammal predisposed to type I diabetes , comprising implanting insulin-producing cells encapsulated in a biologically-compatible membrane. Thus in the absence of working examples or detailed guidance in the specification, the intended uses of the claimed method of preventing onset of Type I diabetes in any mammal, including human are fraught with uncertainties.

Further, the burden of enabling the prevention of a disease (i. e. the need for additional testing) would be greater than that of enabling a treatment due to the need to screen those humans susceptible to such diseases and the difficulty of proof that the administration of the drug was the agent that acted to prevent the condition. Further, the specification does not provide guidance as to how one skilled in the art would go about screening those patients susceptible to Type I diabetes within the scope of the presently claimed invention. Nor is guidance provided as to a specific protocol to be utilized in order to prove the efficacy of the presently claimed compounds

in preventing these disease states. Knip M (Acta Pediatr. Suppl., 1998, V.452, pages 54-62) teaches that currently the state of the art is that successful prevention of type I diabetes has at least two precondition. First, one must be able to identify individuals at increase risk for progression to type I diabetes and second, must have an intervention modality with less severe adverse effects than those associated with disease itself. Total eradication of clinical type I diabetes cannot be expected in the next century, as it is probable that a combination of different interventions will be needed to achieve an optimal effect (see entire document, page 60 in particular). Accordingly, undue experimentation is necessary to determine screening and testing protocols to demonstrate the efficacy of the presently claimed invention.

Thus, Applicant has not provided sufficient guidance to enable one skill in the art to use claimed method of preventing onset of type I diabetes in mammal predisposed to type I diabetes , comprising implanting insulin-producing cells encapsulated in a biologically-compatible membrane in manner reasonably correlated with the scope of the claims. The scope of the claims must bear a reasonable correlation with the scope of enablement. *In re Fisher*, 166 USPQ 18(CCPA 1970) indicates that the more unpredictable an area is, the more specific enablement is necessary in order to satisfy the statute.

In view of the quantity of experimentation necessary, the unpredictability of the art, the lack of sufficient guidance in the specification, the limited working examples, and the limited amount of direction provided given the breadth of the claims, it would take undue trials and errors to practice the claimed invention.

8. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless --

(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 37(c) of this title before the invention thereof by the applicant for patent.

9. Claims 2-5, 7-9 are rejected under 35 U.S.C. 102(e) as being anticipated by US Patent 6,703,017 or by US Patent 5,425764.

US Patent '017 teaches a method of treating and preventing onset of type I diabetes in a mammal comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane (see entire document, Abstract and columns 6, 8, 9 –14 and Example 12 in particular) . US Patent '017 teaches that insulin producing cells are pancreatic islet cells from primary cell source (see columns 8 and 11 in particular). US Patent '017 teaches that pancreatic islet cells are from the same species as the mammal and are implanted interperitoneally into the tissue of a mammal beneath the kidney capsule (see overlapping columns 13-14 and Example 2 in particular). US Patent '017 teaches that encapsulation of said insulin-producing cells in biologically compatible membrane for success of implantation is well known in the art (see column 12 and Example 12 in particular).

US Patent '764 teaches a method of treating and preventing onset of diabetes in a mammal comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane (see entire document, Abstract and overlapping columns 5-6 in particular). US Patent '764 teaches that insulin producing cells are pancreatic islet cells (see column 1 and 4 in particular). US Patent '764 teaches that cells are implanted interperitoneally (see column 5 in particular).

The references teaching anticipates the claimed invention.

10. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(f) or (g) prior art under 35 U.S.C. 103(a).

11. Claims 2-9 are rejected under 35 U.S.C. 103(a) as being unpatentable over US Patent 6,703,017 or by US Patent 5,425764 each and in view of US Patent 5,529,914.

The teaching of US Patent 6,703,017 and US Patent 5,425764 have been discussed, supra.

US Patent 6,703,017 or US Patent 5,425764 does not explicitly teaches that a method of preventing onset of Type I diabetes wherein insulin-producing cells are encapsulated in a conformal coating comprises polyethylene glycol (PEG).

US Patent '914 teaches a new type of biocompatible membrane as a covering to encapsulate biological materials, comprising PEG that is acceptable for implants in mammalian. (see entire document, Abstract in particular). US Patent '914 teaches that various types of cells can be encapsulated in said biocompatible membrane and that said encapsulation will prevent rejection of encapsulated cells during transplantation (see column 10 in particular).

It would have been obvious to a person of ordinary skill in the art at the time the invention was made to apply the teaching of US Patent '914 to those of US Patent ' 017 or US Patent '764 to obtain a claimed method of preventing onset of Type I diabetes wherein insulin-producing cells are encapsulated in a conformal coating comprises polyethylene glycol (PEG).

One of ordinary skill in the art at the time the invention was made would have been motivated to do so, because encapsulation of cells in biologically compatible membrane comprising PEG will prevent rejection of encapsulated cells during transplantation as taught by US Patent '914. Said type of biocompatible membrane can be used to substitute the different type of biocompatible membrane for successful implantation of insulin-producing cells in the method of treating or preventing Type I diabetes taught by US Patent ' 017 or US Patent '764. The strongest rationale for combining references is a recognition, expressly or impliedly in the prior art or drawn from a convincing line of reasoning based on established scientific principles or legal precedent, that some advantage or expected beneficial result would have been produced by their combination. In re Semaker. 217 USPQ 1, 5 - 6 (Fed. Cir. 1983). See MPEP 2144.

From the combined teaching of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

12. No claim is allowed.

13. The lengthy specification has not been checked to the extent necessary to determine the presence of all possible minor errors. Applicant's cooperation is requested in correcting any errors of which Applicant may become aware in the specification.

14. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michail Belyavskyi whose telephone number is 571/272-0840. The examiner can normally be reached Monday through Friday from 9:00 AM to 5:30 PM. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan can be reached on 571/272-0841.

The fax number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Michail Belyavskyi, Ph.D.
Patent Examiner
Technology Center 1600
December 10, 2004

Christina Chan

CHRISTINA CHAN
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. LATTA.002C3	APPLICATION NO. 10/660,924
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (USE SEVERAL SHEETS IF NECESSARY)	APPLICANT PAUL P. LATTA	
	FILING DATE September 12, 2003	GROUP 1632

U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE (IF APPROPRIATE)
MB	1.	4,298,002	11/03/81	RONEL et al.			
	2.	4,353,888	10/12/82	SEFTON			
	3.	4,378,016	03/29/83	LOEB			
	4.	4,673,566	06/16/87	GOOSEN et al.			
	5.	4,689,293	08/25/87	GOOSEN et al.			
	6.	4,696,288	09/29/87	COCHRUM			
	7.	4,806,355	02/21/89	GOOSEN et al.			
	8.	4,892,538	01/09/90	AEBISCHER et al.			
	9.	4,902,295	02/20/90	WALTHALL et al.			
	10.	4,997,443	03/05/91	WALTHALL et al.			
	11.	5,182,111	01/26/93	AEBISCHER et al.			
	12.	5,262,044	11/16/93	BAE et al.			
	13.	5,290,684	03/01/94	KELLY			
	14.	5,529,914	06/25/96	HUBBELL et al.			
MB	15.	5,425,764	06/20/95	FOURNIER et al.			

FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
MB	16.	A2 0,147,939	10/07/85	EPO				
	17.	A1 2,034,641	28/05/92	CANADA				
	18.	WO 92/19195	12/11/92	PCT				
	19.	WO 95/03082	02/02/95	PCT				
MB	20.	0 536 807 A1	04/02/87	EP				

EXAMINER INITIAL	OTHER DOCUMENTS (INCLUDING AUTHOR, TITLE, DATE, PERTINENT PAGES, ETC.)
	21. Aebischer, P. et al., "LONG-TERM CROSS-SPECIES BRAIN TRANSPLANTATION OF A POLYMER-ENCAPSULATED DOPAMINE-SECRETING CELL LINE" *Experimental Neurology (1991) 111:269-275*
	22. Aebischer, P. et al., "TRANSPLANTATION OF POLYMER ENCAPSULATED NEUROTRANSMITTER SECRETING CELLS: EFFECT OF THE ENCAPSULATION TECHNIQUE" *Journal of Biomechanical Engineering (1991) 113:178-183*

EXAMINER	DATE CONSIDERED 12/9/07

*EXAMINER: INITIAL IF CITATION CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP 609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED, INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. LATTA.002C3	APPLICATION NO. 10/660,924
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (USE SEVERAL SHEETS IF NECESSARY)	APPLICANT PAUL P. LATTA	
	FILING DATE September 12, 2003	GROUP 1632

EXAMINER INITIAL		OTHER DOCUMENTS (INCLUDING AUTHOR, TITLE, DATE, PERTINENT PAGES, ETC.)
	23.	Bartlett, S.T. et al., "COMPOSITE KIDNEY-ISLET TRANSPLANTATION PREVENTS RECURRENT AUTOIMMUNE BETA-CELL DESTRUCTION" *Surgery (1993) 114:211-217*
	24.	Buchser, et al., "IMMUNOISOLATED XENOGENIC CHROMAFFIN CELL THERAPY FOR CHRONIC PAIN. INITIAL CLINICAL EXPERIENCE" *Anesthesiol., (1996) 85:1005-1012*
	25.	Chicheportiche, D. et al., "IN VITRO KINETICS OF INSULIN RELEASE BY MICROENCAPSULATED RAT ISLETS: EFFECT OF THE SIZE OF THE MICROCAPSULES" *Diabetologia (1988) 31:54-57*
	26.	Colton, C.K. (1995), "IMPLANTABLE BIOHYBRID ARTIFICIAL ORGANS" *Cell Transplantation 4(4):415-436.*
	27.	Dixit, V. et al., "A MORPHOLOGICAL AND FUNCTIONAL EVALUATION OF TRANSPLANTED ISOLATED ENCAPSULATED HEPATOCYTES FOLLOWING LONG-TERM TRANSPLANTATION IN GUNN RATS" *Biomat. Art. Cells & Immob. Biotech. (1993) 21(2):119-133*
	28.	Gao, E-K et al., "T CELL CONTACT WITH Ia ANTIGENS ON NONHEMOPOIETIC CELLS IN VIVO CAN LEAD TO IMMUNITY RATHER THAN TOLERANCE" *J. Exp. Med (1991)174:435-446*
	29.	Gilbert, J.C. et al., "CELL TRANSPLANTATION OF GENETICALLY ALTERED CELLS ON BIODEGRADABLE POLYMER SCAFFOLDS IN SYNGENEIC RATS" *Transplantation (1993) 56(2):423-427*
	30.	Hansan, et al., "EVIDENCE THAT LONG-TERM SURVIVAL OF CONCORDANT XENOGRAFTS IS ACHIEVED BY INHIBITION OF ANTISPECIES ANTIBODY PRODUCTION" *Transplantation, (1992) 54:408-413*
	31.	Hill, R.S. et al., "MEMBRANE ENCAPSULATED ISLETS IMPLANTED IN EPIDIDYMAL FAT PADS CORRECT DIABETES IN RATS" *Cell Transplantation (1992) 1(213):132 p. 168*
	32.	Hoffman, D. et al., "TRANSPLANTATION OF A POLYMER-ENCAPSULATED CELL LINE GENETICALLY ENGINEERED TO RELEASE NGF" *Experimental Neurology (1993) 122:100-106*
	33.	Husby, s. et al., "ORAL TOLERANCE IN HUMANS. T CELL BUT NOT B CELL TOLERANCE AFTER ANTIGEN FEEDING" *J. Immunol., (1994)152:4663-4670*
	34.	Kneteman, N.M. et al., "ISOLATION AND CRYOPRESERVATION OF HUMAN PANCREATIC ISLETS" *Transplantation Proceedings (1986) XVIII(1):182-185*
	35.	Lacy, P.E. et al., "MAINTENANCE OF NORMOGLYCEMIA IN DIABETIC MICE BY SUBCUTANEOUS XENOGRAFTS OF ENCAPSULATED ISLETS" *Science (1991) 254:1782-1784*
	36.	Lanza, R.P. et al., "XENOTRANSPLANTATION OF CANINE, BOVINE, AND PORCINE ISLET" *PNAS USA (1991) 88:11100-11104.*
	37.	Lanza, R.P. et al., "TRANSPLANTATION OF ENCAPSULATED CANINE ISLETS INTO SPONTANEOUSLY" *Endocrinology (1992), 131(2):637-642*
	38.	Liu, H. et al., "EXPRESSION OF HUMAN FACTOR IX BY MICROENCAPSULATED RECOMBINANT FIBROBLASTS" *Human Gene Therapy (1993) 4:291-301*
	39.	Lum, Z. et al., "PROLONGED REVERSAL OF DIABETIC STATE IN NOD MICE BY XENOGRAFTS OF MICROENCAPSULATED RAT ISLETS" *Diabetes (1991) 40:1511-1516*
	40.	Nossal, G.J.V. "IMMUNOLOGICAL TOLERANCE" in: Fundamental Immunology, Second Edition, edited by W.E. Paul, Raven Press, New York, pp 571-585 (1989)
	41.	Osband, ME et al., "PROBLEMS IN THE INVESTIGATIONAL STUDY AND CLINICAL USE OF CANCER IMMUNOTHERAPY" *Immunological Today, (1990) 11(6):193-195*
	42.	Posselt, A.M. et al., "INDUCTION OF DONOR-SPECIFIC UNRESPONSIVENESS BY INTRATHYMIC ISLET TRANSPLANTATION" *Science (1990) 249:1293-1295*
	43.	Posselt, A.M. et al., "INTRATHYMIC ISLET TRANSPLANTATION IN THE SPONTANEOUSLY DIABETIC BB RAT" *Ann. Surg. (1991) 214(4):363-373*

EXAMINER	DATE CONSIDERED

*EXAMINER: INITIAL IF CITATION CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP 609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Latta, Paul P.
Appl. No.	:	10/660,924
Filed	:	September 12, 2003
For	:	PREVENTION OF DIABETES THROUGH INDUCTION OF IMMUNOLOGICAL TOLERANCE
Examiner	:	Belyavski, Michail A.
Group Art Unit	:	1644

AMENDMENT AND RESPONSE TO OFFICE ACTION

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to Office Action mailed December 16, 2004, and interview conducted March 21, 2005 please amend the above-identified application as follows:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Summary of Interview begins on page 3 of this paper.

Remarks/Arguments begin on page 4 of this paper.

AMENDMENTS TO THE CLAIMS

1. **(Canceled)**

2. **(Currently amended)** A method of preventing onset of Type I diabetes in a mammal predisposed to Type I diabetes, comprising implanting a dose of insulin-producing cells encapsulated in a biologically-compatible membrane into an implantation site in said mammal prior to onset of Type I diabetes, wherein said dose is at least one order of magnitude less than that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes.

3. **(Previously presented)** The method of Claim 2, wherein said cells are from a primary cell source.

4. **(Previously presented)** The method of Claim 3, wherein said cells are pancreatic islet cells.

5. **(Previously presented)** The method of Claim 2, wherein said cells are encapsulated in a conformal coating.

6. **(Previously presented)** The method of Claim 5, wherein said conformal coating comprises polyethylene glycol (PEG).

7. **(Previously presented)** The method of Claim 2, wherein the insulin-producing cells are from the same species as the mammal.

8. **(Previously presented)** The method of Claim 2, wherein Type I diabetes is prevented without continuous immunosuppression.

9. **(Previously presented)** The method of Claim 2, wherein the cells are implanted intraperitoneally.

SUMMARY OF INTERVIEW

Exhibits and/or Demonstrations

Experimental data showing that implanting a tolerizing (sub-therapeutic) dose of encapsulated insulin-producing cells into NOD mice prior to the animals developing diabetes protected these animals from diabetes for the rest of their natural lives as shown by their normoglycemia and lack of insulitis.

Identification of Claims Discussed

2-9

Identification of Prior Art Discussed

USP 6,703,017 and 6,425,764

Proposed Amendments

The Applicant proposed to specify the dose of the insulin-producing cells implanted to prevent diabetes Type I.

Principal Arguments and Other Matters

The Applicant argued that claims 2-9 are novel over USP 6,703,017 and 6,425,764.

Results of Interview

The Examiner insisted that the data presented by the inventor showing prevention of Type I diabetes in non-obese diabetic (NOD) mice was not sufficient to show a method of preventing type I diabetes in human. According to the Examiner, only clinical data from human trials could be used for this purpose.

REMARKS

Claim 2 has been amended. Support for the amendment can be found in the Specification as filed, for example, page 9, lines 9-11, page 12, line 26-page 13, line 4; page 20, lines 4-8; and page 25, lines 14-16. Therefore, no new matter has been introduced by this amendment. The following addresses the substance of the Office Action.

1. Title of the invention is descriptive

The current title of the application is: "Prevention of Diabetes through Induction of Immunological Tolerance". This title accurately reflects the claimed invention, and distinctly points out what is claimed in the application.

2. References in IDS not found in File

Applicant has resubmitted the references previously submitted in the parent application.

3. Compliance with the Written Description Requirement

The Examiner rejected Claims 2-9 under the written description requirement of 35 U.S.C. §112, first paragraph. The Examiner pointed out that the specification must describe the invention in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. In accordance with the written description guidelines of MPEP 2163, "[p]ossession may be shown in a variety of ways including . . . by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention."

Here, the specification is quite clear in showing the distinguishing identifying characteristics of the subject matter of Claims 2-9. In particular, all of the features of Claim 2 are specifically described in the specification as filed at page 3, line 28 through page 4, line 19. Additional description of the specific features of these claims is set forth at page 4, lines 3-4; page 10, lines 12-16, and page 19, lines 1-14. One skilled in the art reviewing these portions of the specification would have no doubt that the Applicants had possession of the invention. Therefore, Applicant asserts that Claims 2-9 are supported in the Specification as filed and are in compliance with 35 U.S.C. §112, first paragraph.

4. Compliance with the Enablement Requirement

The Examiner believes that the specification does not reasonably provide enablement for a method of preventing onset of Type I diabetes in a mammal comprising implanting insulin-

producing cells encapsulated in a biologically compatible membrane. The reasons the Examiner stated for the rejection are: **A)** unpredictability of the prevention of diabetes in human from the murine data; **B)** need for screening for susceptible individuals; **C)** need for undue experimentation to determine screening and testing protocols to demonstrate the efficacy of the claimed invention. Each of these reasons is addressed below:

A. Reasonable predictability of the prevention of diabetes in human from *in vivo* data obtained in NOD mice.

As discussed above in connection with compliance with the written description requirement, the specification describes every feature of the claimed invention. One skilled in the art would have no difficulty carrying out the steps for making and using the invention based on this description. To establish that one actually carrying out these steps could successfully achieve the claimed result, i.e. prevention of diabetes, Applicant submitted a Declaration of David Scharp showing that NOD mice receiving the treatment described in the specification were prevented from becoming diabetic. Nevertheless, the Examiner is questioning the value of such evidence based on alleged lack of predictability of the prevention of diabetes in human from *in vivo* data obtained in the NOD mice.

However, the Examiner is setting forth a much stricter standard than required by law. MPEP 2107.03 establishes the following:

> Evidence does not have to be in the form of data from an art-recognized animal model for the particular disease or disease condition to which the asserted utility relates. Data from any test that the applicant reasonably correlates to the asserted utility should be evaluated substantively. Thus, an applicant may provide data generated using a particular animal model with an appropriate explanation as to why that data supports the asserted utility. The absence of a certification that the test in question is an industry-accepted model is not dispositive of whether data from an animal model is in fact relevant to the asserted utility. Thus, if one skilled in the art would accept the animal tests as being reasonably predictive of utility in humans, evidence from those tests should be considered sufficient to support the credibility of the asserted utility."

In the present case, the animal tests submitted by Applicant have been established as reasonably predictive by those skilled in the art. The NOD (non-obese diabetic) mouse is the standard animal model for conducting research on Type I diabetes and other autoimmune disease. The NIH recognize the NOD mouse as the model animal for diabetes and maintains a research colony and data base on these animals for researchers. The NIH state "The NOD mouse, which

spontaneously develops type 1 diabetes, is a valuable animal model that is used extensively in research exploring the etiology, prevention, and treatment of this disease. It is a vital research tool for testing promising prevention and treatment strategies at the preclinical level." (http://www.niaid.nih.gov/dait/NODmice.htm, copy attached herein).

Moreover, Hanninen et al. (2003 "Development of new strategies to prevent type I diabetes: the role of animal models" *Annals of Medicine* **35**:546-563, copy attached herein) states: "The non-obese diabetic (NOD) mouse is the most widely used animal model of T1DM. [...] research in non-obese diabetic mice has led to the discovery of new strategies of diabetes prevention that are now in human clinical trials". The authors further present a whole list of current clinical trials based on strategies developed in NOD mice.

Additionally, the US PTO has also previously accepted the predictability of the results of prevention of diabetes in NOD mice and allowed claims to a method of protecting against the development of autoimmune diabetes in a susceptible subject (USP 6,841,152).

In addition, the Second Declaration of David Scharp under 37 CFR 1.132 submitted herewith states that the NOD mouse is the only animal model for human autoimmune, Type I diabetes because it is the only available model reasonably predictive of human disease. The same Declaration also shows that using the "one to two orders of magnitude" criteria for calculating the dose for tolerizing the experimental animals was successful in preventing the autoimmune disease under study – Type I diabetes. The NOD mice were implanted with 50 to 150 islets, which resulted in prevention of diabetes in these animals.

While it is true that questions have been raised whether the NOD model is absolutely predictive of treatment of humans, such an absolute correlation with human disease is not required to support enablement. MPEP 2107.03 further provides:

> The applicant does not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty, nor does he or she have to provide actual evidence of success in treating humans where such a utility is asserted. Instead, as the courts have repeatedly held, all that is required is a reasonable correlation between the activity and the asserted use.

Therefore, using the proper standard set forth in the MPEP, the evidence provided by Applicant clearly supports that one skilled in the art would accept the NOD model as reasonably correlating to the condition in human.

B. Screening for susceptible individuals is well-known and routine

The Examiner also objected to the enablement provided by the specification based on an alleged failure to disclose how to screen for susceptible individuals. However, the specification as filed on page 19, lines 9-12, provides information that "in diabetes, the use of immune marker autoantibodies to establish preclinical diabetes has been well studied" The specification cites Palmer, *Diabetes Rev.* 1(1):104-116, 1993 in support of this statement. In addition, Applicant provides herewith several references to support the position that screening for individuals susceptible to developing type I diabetes has been well-established in the art at the time the invention was made: Bonifacio et al. 1995 "Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity", *Diabetologia* **38**:816-22; Lee et al. 1995 "Relationships among 64k autoantibodies, pancreatic beta-cell function, HLA-DR antigens and HLA-DQ genes in patients with insulin-dependent diabetes mellitus in Korea", *Korean J. Intern. Med.* **10**:1-9; Bingley et al. 1994 "Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives", *Diabetes* **43**:1304-10; Zimmet et al. 1994 "Autoantibodies to glutamic acid decarboxylase and insulin in islet cell antibody positive presymptomatic type 1 diabetes mellitus: frequency and segregation by age and gender", *Diabet Med.* **11**:866-71; Christie et al. 1994 "Antibodies to islet 37k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity", *Diabetes* **43**:1254-9; Tuomilehto et al. 1994 "Antibodies to glutamic acid decarboxylase as predictors of insulin-dependent diabetes mellitus before clinical onset of disease", *Lancet* **343**:1383-5; Zimmet et al. 1994 "Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency", *Diabet Med.* **11**:299-303, copies of which are submitted herewith.

"The specification need not disclose what is well known in the art." *See, e.g., In re Buchner,* 18 USPQ2d 1331 (Fed. Cir. 1991). Thus, there was no requirement for Applicants' specification to contain a thorough description of the well known techniques that were well-established as of the effective filing date. Accordingly, one skilled in the art would have no difficulty identifying suitable subjects for the treatment of the present invention.

C. Experimentation to determine screening and testing protocols to demonstrate the efficacy of the claimed invention in not undue

The Examiner also believed that undue experimentation would be required to determine screening and testing protocols. However, as established in both Declarations of David Scharp, M.D., testing protocols have already been established with minimum difficulty. As apparent from the claim, the goal of the invention is to prevent diabetes, i.e. maintain normoglycemia. Methods for determining whether normoglycemia is present have been exceedingly well known for many years; thus, only routine blood glucose monitoring would be required to demonstrate the efficacy of the claimed invention.

MPEP 2164.01(c) establishes that in order to meet the enablement requirement, one skilled in the art need only be able to discern an appropriate dosage or method of use without undue experimentation based on knowledge of compounds having similar physiological or biological activity. Here, the Specification at page 12, lines 26-30 clearly indicates that a dose of implanted insulin-producing cells to induce tolerance is one or two orders of magnitude less than a full dose of implant which provides adequate insulin production for normoglycemia. The full dose for achieving normoglycemia has been well worked out for many years. Accordingly, no difficulty would be had in obtaining the correct dose for any given individual. Accordingly, no undue experimentation would be required to practice the claimed invention.

5. Compliance with 35 U.S.C. 102

The Examiner rejected Claims 2-5, 7-9 as anticipated by US Patent 6,703,017 or by US Patent 5,425,764. In order to anticipate, the reference must teach each and every element of the claim. The '017 patent describes implanting insulin-producing cells in a dose of about 8,000-12,000 islets/kg of patient's body weight (col. 14, lines 7-9) to create a pancreas-like structure in a human patient. Therefore the implant in USP '017 is designed to treat diabetes by creating a live "insulin pump" in the body. Furthermore, Example 12 of USP '017 describes implanting 5,000 islets per NOD mouse which has developed diabetes (this dose equals 200,000 islet/kg of body weight), which resulted in normoglycemia in these animals. The '017 patent does not teach implanting a dose of insulin-producing cells encapsulated in a biologically-compatible membrane prior to onset of Type I diabetes, wherein said dose is at least one order of magnitude less than that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes. The "017 patent only concerns itself with treating diabetes once established with

implanting islets, not preventing diabetes from becoming established by implanting a small, sub-therapeutic dose of encapsulated cells. Therefore, Claim 2 as currently amended, as well as claims dependent on Claim 2 are not anticipated by US Patent 6,703,017.

US Patent 5,425,764 describes a method of using an implantable bioartificial pancreas device containing insulin-secreting islets, to supply an exogenous source of insulin to treat the symptoms of diabetes. Accordingly, the '764 patent requires implantation of a curative dose of insulin-secreting cells, i.e. the dose necessary to achieve normoglycemia. As such the '764 Patent does not teach implanting a dose of insulin-producing cells encapsulated in a biologically-compatible membrane prior to onset of Type I diabetes, wherein said dose is at least one order of magnitude less than that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes. The '764 patent is focused on a therapy for diabetes by implanting a large quantity of islets, and does not consider preventing diabetes from occurring by implanting a small dose of encapsulated islets. Therefore, Claim 2 as currently amended as well as claims dependent on Claim 2 are not anticipated by the '764 Patent.

Thus, Claims 2-5, 7-9 are in compliance with 35 U.S.C. § 102.

6. Compliance with 35 U.S.C. 103(a)

The Examiner rejected Claims 2-9 under 35 U.S.C. 103(a) as unpatentable over US Patent 6,703,017 or by US Patent 5,425764 in view of US Patent 5,529,914. However, pursuant to MPEP 2143, in order to establish a *prima facie* case of obviousness three requirements must be met: First there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

In the case of the present invention, the cited references fail to suggest all of the claim limitations. The novelty of Claims 2-9 over US Patent 6,703,017 and US Patent 5,425,764 is discussed above. As discussed above, neither of these patents teaches, or even suggests, a method involving implantation of a sub-curative dose of insulin-secreting cells. US Patent 5,529,914 discloses a method of encapsulating cells, but it fails to cure the deficiencies of US

Patent 6,703,017 and US Patent 5,425764. Therefore, Claims 2-9 are in compliance with 35 USC §103(a).

CONCLUSION

Applicants have endeavored to address all of the Examiner's concerns as expressed in the outstanding Office Action. Accordingly, amendments to the claims, the reasons therefor, and arguments in support of the patentability of the pending claim set are presented above. In light of the above amendments and remarks, reconsideration and withdrawal of the outstanding rejections is specifically requested. If the Examiner finds any remaining impediment to the prompt allowance of these claims that could be clarified with a telephone conference, the Examiner is respectfully requested to initiate the same with the undersigned.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: April 29, 2005

By: _____

Marina L. Gordey
Registration No. 52,950
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Customer No. 20,995
(805) 547-5580

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LATTA.002C3 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Latta, Paul P.
Appl. No.	:	10/660,924
Filed	:	September 12, 2003
For	:	PREVENTION OF DIABETES THROUGH INDUCTION OF IMMUNOLOGICAL TOLERANCE
Examiner	:	Belyavski, Michail A.
Group Art Unit	:	1644

SECOND DECLARATION OF DAVID SCHARP, M.D.
UNDER 37 C.F.R §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, David Scharp, M.D., am Chief Scientific Officer of Novocell, Inc. At Novocell I am actively engaged in research related to development of treatment of diabetes using encapsulated insulin-producing cells.

2. I have extensive experience in the field of the claimed invention as indicated in the attached Curriculum Vitae provided herewith as Exhibit A.

3. There are two potential animal models for studying Type I diabetes, the BB rat and the NOD mouse. The BB rat, while developing diabetes, has a multitude of immunologic disorders that makes it more of a model for immune deficiency than diabetes. The BB rat is no longer considered an acceptable model for studying human autoimmune diabetes.

4. The NOD mouse is therefore, the only animal model for human autoimmune, Type I diabetes in that it is predictive of human disease. Its lymphocytes spontaneously begin attacking

its insulin-producing pancreatic beta cells soon after birth. Looking at the histology of the pancreas of these mice during the autoimmune process, one finds early that most islets are infiltrated with immune cells that are destroying islets. As the process continues towards the diabetic phase, almost all of the beta cells are destroyed leaving smaller than normal islets with residual inflammation that continues to destroy the new islets that are stimulated to develop due to the failing islet mass. This ongoing destruction of the insulin-producing pancreatic beta cells continues and progresses for 15 to 32 weeks until a sufficient number of beta cells are destroyed to cause the clinical onset of Type I diabetes in NOD mice. Examining living NOD mice prior to development of clinical diabetes, including monitoring their blood glucose levels, one would have no clue that this autoimmune process is actively destroying their pancreatic beta cells. Yet, if one examines their blood for anti-islet protein antibodies, one can clearly identify those animals that will eventually lose blood glucose control and develop clinical diabetes. This situation is identical for human Type I diabetes in that patients at high risk for developing Type I diabetes are tested for the presence of specific auto-antibodies. The number and titers of these specific antibodies can now predict with >90% certainty which of these patients with ongoing autoimmune destruction of their beta cells will actually develop clinical Type I diabetes within 5 years.

5. By following a large number of patients over time, it has been determined that there are clearly many years available in which to intervene in the auto-immune process prior to the development of clinical diabetes in an attempt to prevent it, if there were an effective prevention method available. Six drugs have been tried in patients with documented evidence of autoimmune diabetes to determine if human diabetes can be prevented. To date, such drug treatments have not been effective in preventing clinical diabetes in humans.

6. Our approach is totally different in that we are not giving drugs to prevent diabetes. Instead, we are implanting a very small fraction of encapsulated islet allografts into these NOD mice prior to the development of clinical diabetes in order to stop the onset of clinical, Type I diabetes. This fraction of encapsulated islets is so small it would have no effect on controlling blood glucose if given after diabetes had begun and therefore, is not a treatment for diabetes. We believe that the continuous release of donor antigens from the small fraction of encapsulated

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islets that are not destroyed by the host, alters the auto-immune response in a way that the host no longer considers the islets to be foreign. In this way, the immune attack on the islets within the pancreas stops prior to the onset of clinical diabetes and thus prevents clinical diabetes.

7. In my previous declaration, filed on April 1, 2004, I presented the data from the NOD mouse study on Prevention of Type I diabetes. This data has now been summarized into two bar graphs to allow an easier analysis.

8. Islets from mouse strain C57Bl/6 were encapsulated by polyethylene glycol conformal coating as described in USP 5,529,914. This patent was incorporated by reference into the specification of the application captioned above. The conformally-coated islets were implanted by intraperitoneal injection into NOD mice. The experiment had two variables under study – time of implantation of the encapsulated islets (4, 8, and 12 weeks) and number of islets implanted (50, 100 and 150 islet equivalents, IEQ).

9. **Exhibit 1** shows the data for all animals grouped by time of implantation. Implantation at 4 weeks showed the best results with diabetes being prevented in 60% of the treated animals, as compared to the control animals with none having diabetes prevented.

10. **Exhibit 2** shows the data for all animals grouped by number of islets implanted. A dose of 50 IEQ produced the best results with diabetes being prevented in 60% of the treated animals. All the control animals developed diabetes.

11. **Exhibit 3** demonstrates that in the control recipients (NOD mice not implanted with encapsulated islet cells), the autoimmune destruction of the pancreatic islets is very complete with small shrunken islets remaining with continuing evidence of lymphocytes destroying any new islets that are formed.

12. **Exhibits 4 & 5** demonstrate that in those recipients that were prevented from developing diabetes after implanting the small quantity of encapsulated islets, very large islets (many times their normal size) are present, without evidence of host lymphocyte destruction. This means that the normal process in the mouse to replace lost islets has been successful to the point of preventing diabetes from destroying all of the newly formed islet cells.

13. This demonstrates that the method of the present invention prevents type I diabetes.

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14. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: April 28, 2005 By: _____
David Scharp

1678364
042105

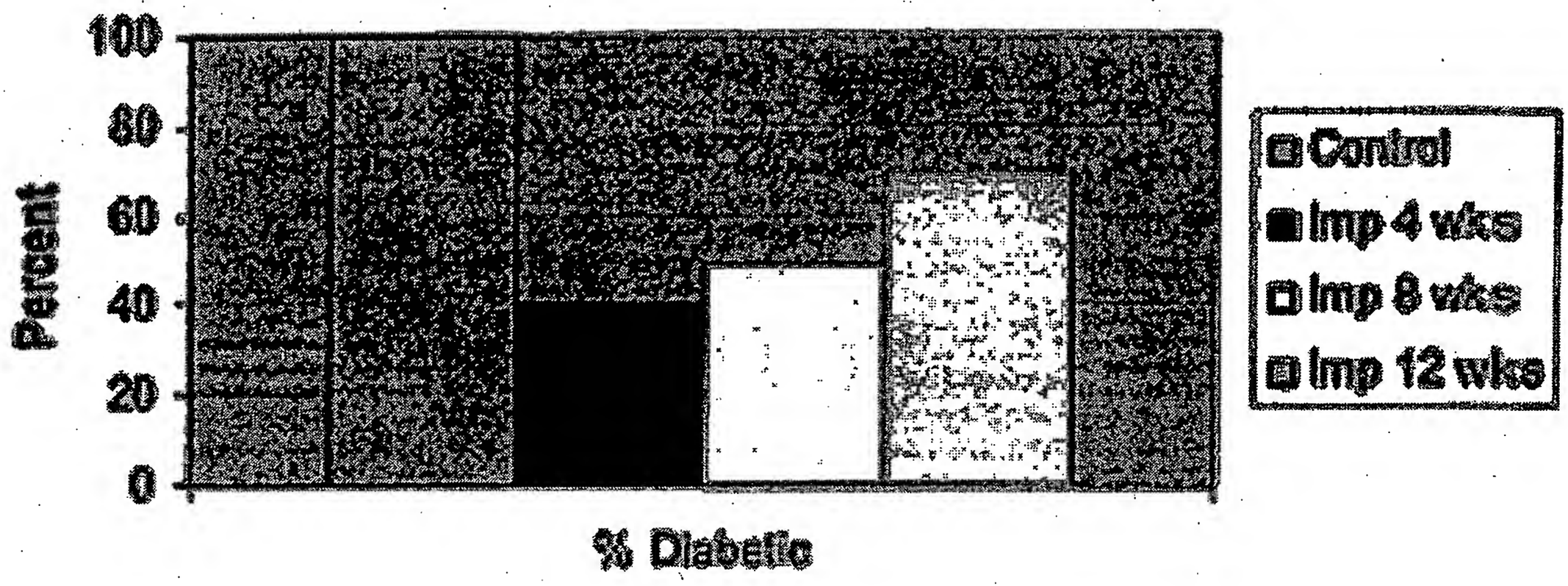
Prevention of Diabetes in NOD Mice with Implants of Encapsulated Islet Allografts
100
80
60
40
20
0
Percent
% Diabetic
Control
Imp 4 wks
Imp 8 wks
Imp 12 wks

Exhibit 1

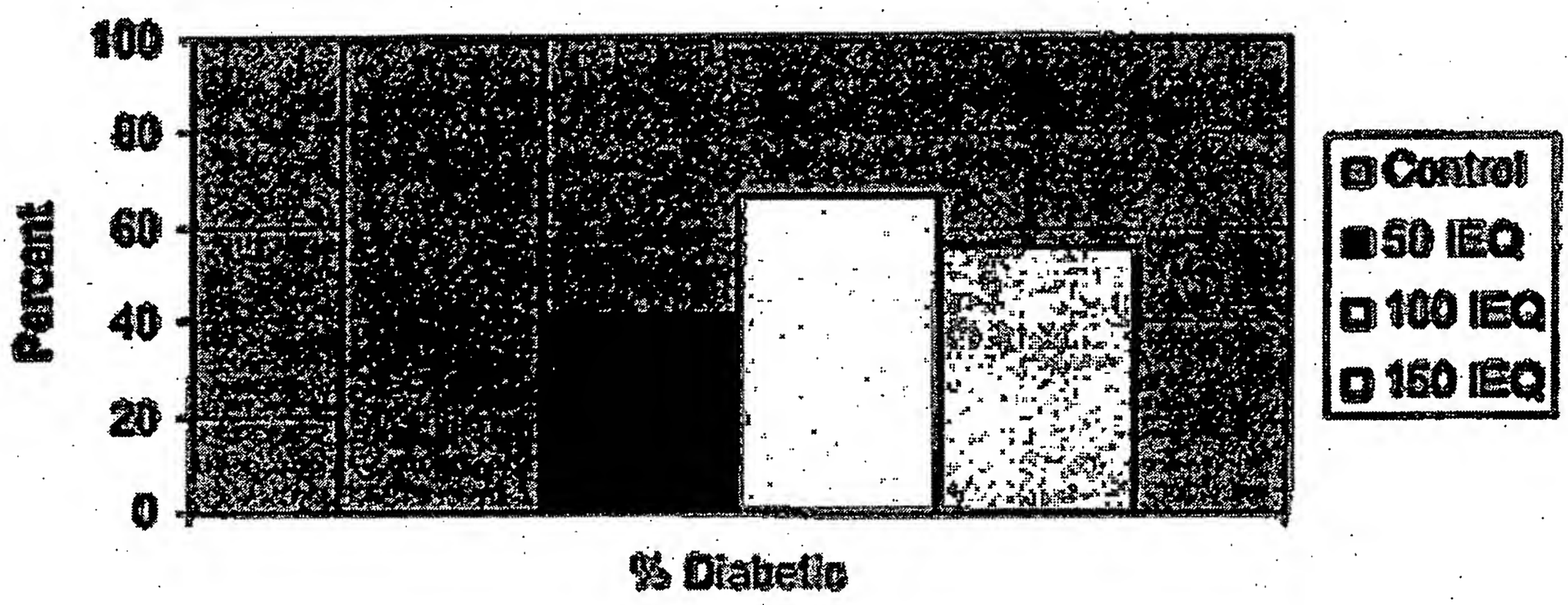
Prevention of Diabetes in NOD Mice with Implants of Encapsulated Islet Allografts
100
80
60
40
20
0
Percent
% Diabetic
Control
50 IEQ
100 IEQ
150 IEQ

Exhibit 2

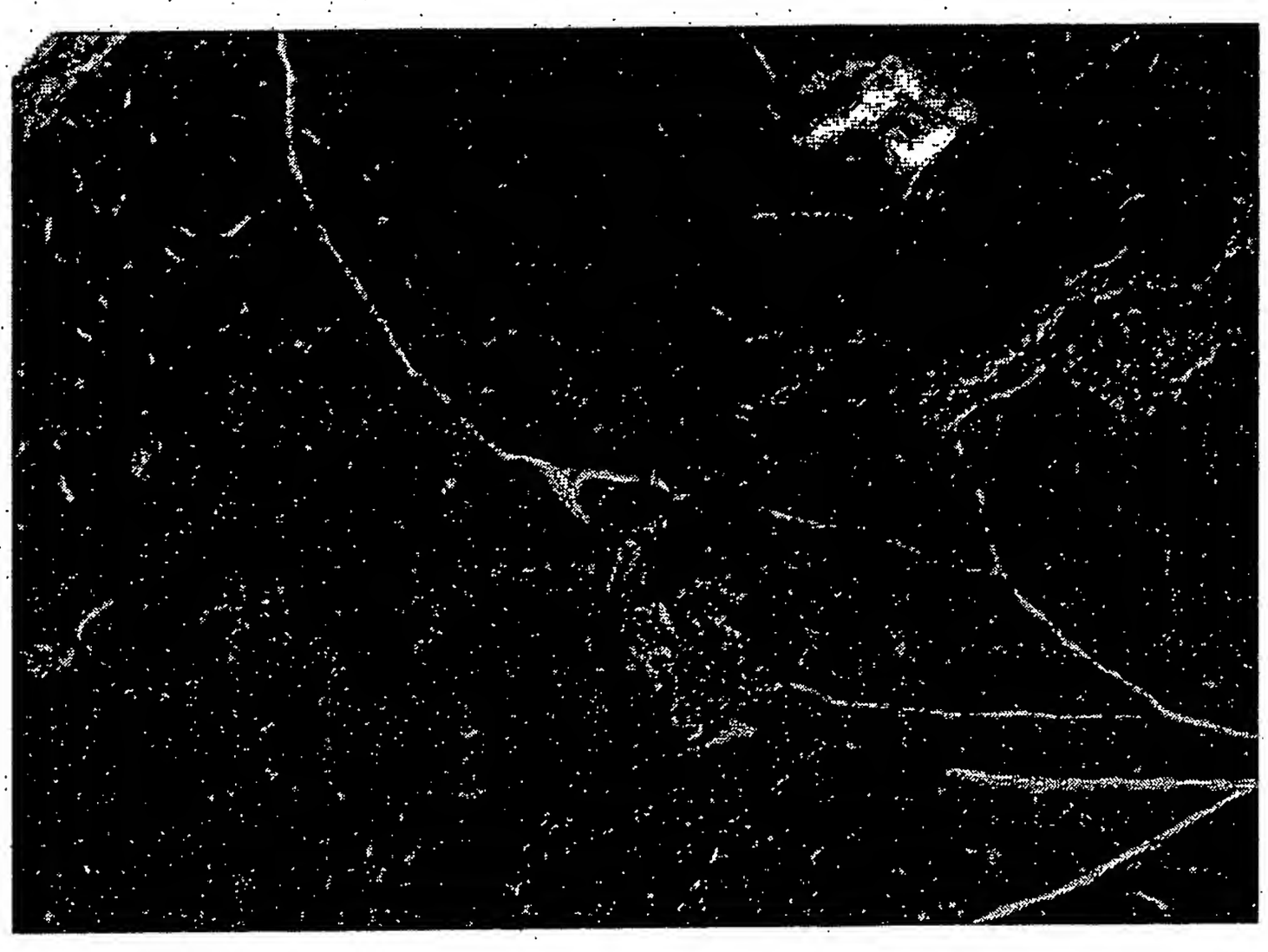

Exhibit 3

Exhibit 4

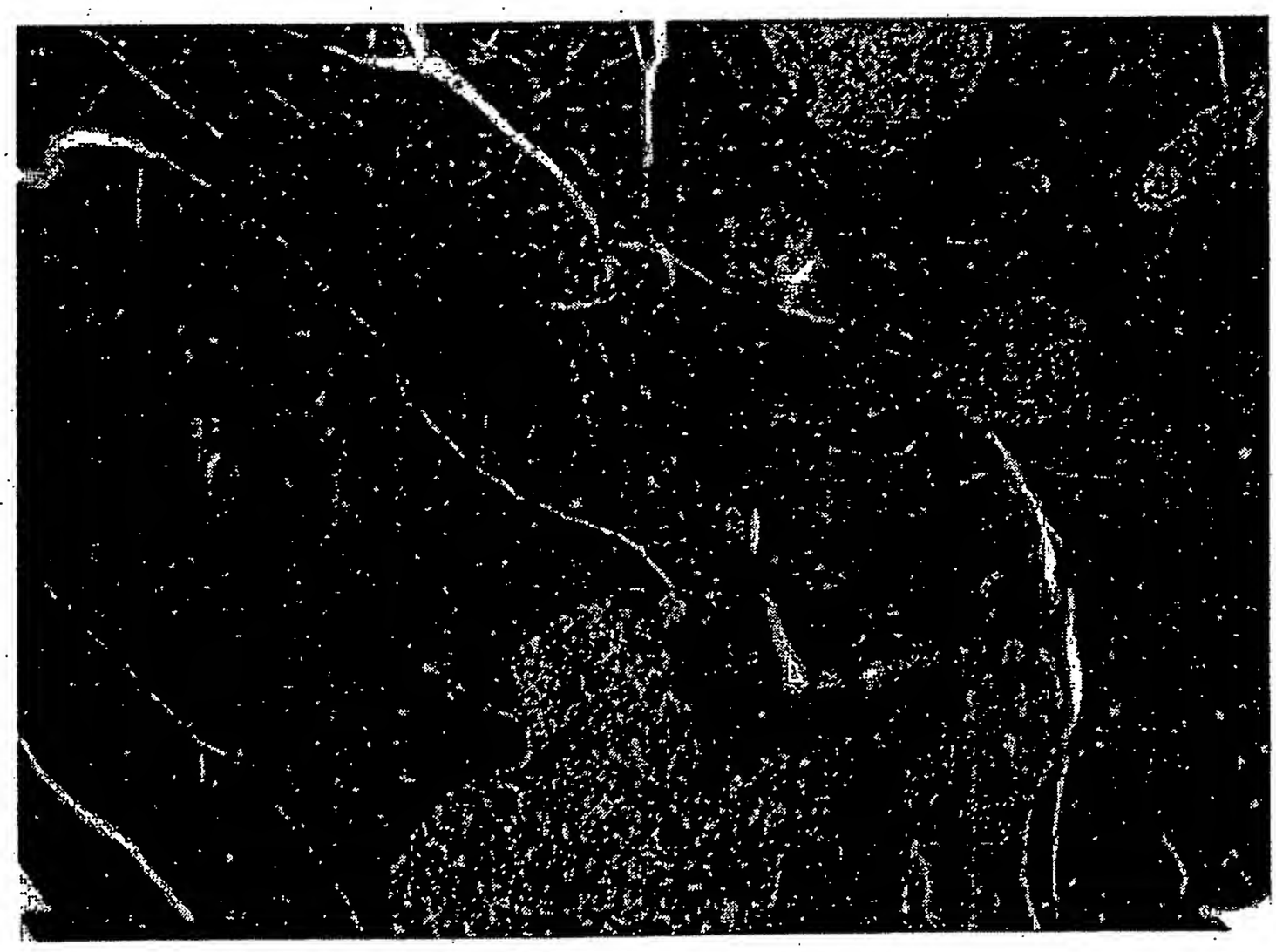

Exhibit 5

CURRICULUM VITAE

David William Scharp, M.D.

Born: July 5, 1945
Washington, Illinois

Social Security Number: 327-36-7524

Marital Status: Wife: Jeanette

Children: Kevin Scharp
Daniel Scharp
Anna Scharp
David Bondurant
Melissa Bondurant

Pre-Medical Education: University of Missouri
Columbia, Missouri
1963-1966

Medical Education: Washington University School of Medicine
St. Louis, Missouri
1966-1970

Graduate Hospital Experience:

Intern in Surgery
7/1/70-6/30/71
Barnes Hospital/Washington University

Surgical Resident
7/1/71-6/30/72 & 7/1/74-6/30/76
Barnes Hospital/Washington University

Surgical Research Fellow
7/1/72-6/30/74
Washington University - Department of Surgery

Academic Positions:

Assistant Professor of Surgery
1976-1983
Washington University School of Medicine

Associate Professor of Surgery
1983-1991
Washington University School of Medicine

Professor of Surgery
1991-President
Washington University School of Medicine

Leave of Absence
1/1/94-7/1/95

Commercial Positions:

Novocell, Inc.
Chief Scientific Officer

Neocrin Company
Chief Scientific Officer 1/1/94-7/31/99
Executive Vice President of Medical Affairs 1/1/94-7/31/99
Executive Vice President for Research 1/1/94-11/1/95
Executive Vice President, Research and Development 11/1/95-President

McDonnell Douglas Corporation 1984-1987
Contractual Research Investigator
Electrophoretic Separation of Islet Cells

Cytotherapeutics, Inc. 1989-1993
Founding Scientist
Scientific Advisory Board Member
Contractual Research Investigator

Patents:

"Islet Isolation Process"
DE191613T – 1987
DE3650662D – 1998
EP0191613 – 1986
EP0191613 – 1989
EP0191613 – 1997
JP61183226 – 1986
US4868121 – 1989
US5322790 – 1994

"Method to Isolate Clusters of Cell Sub-Types from Organs"
AU1934988 – 1989
CA1340406 – 1999
EP0382727 – 1990
EP0382727 – 1991
JP2504222T – 1990
US5079160 – 1992
WO8809667 – 1988

"Implantable Biocompatible Immunoisolatory Vehicle for Delivery of Selected Therapeutic Products"
AT156344T – 1997
AU666118 – 1996
AU682796 – 1997
AU2004192 – 1992
AU3902095 – 1996
CA2109085 – 1992
CA2109085 – 2003
DE69221484D – 1997
DE69221484T – 1998
DK585368T – 1998
EP0585368 – 1994
EP0585368 – 1994
EP0585368 – 1997
ES2107537T – 1997
FI934545 – 1993
FI934545D – 1993
GR3025301T – 1998
HK1001832 – 1998
JP6507412T – 1994
NO308198B – 2000
NO933842 – 1993
SG47470 – 1998

US5798113 – 1998
US5800828 – 1998
US5871767 – 1999
US6083523 – 2000
US6322804 – 2001
US2002150603 – 2002
WO9219195 – 1992

"Methods for Coextruding Immunoisolatory Implantable Vehicles with a Biocompatible Jacket and a Biocompatible Matrix Core"
US5800829 – 1998

"Methods for Treating Diabetes by Delivering Insulin from Biocompatible Cell Containing Devices"

US5869077 – 1999

"Methods for Making Immunoisolatory Implantable Vehicles with a Biocompatible Jacket and a Biocompatible Matrix Core"
US5834001 – 1998
US5874099 –1999

"Use of Pouch for Implantation of Living Cells"
AU4788993 – 1994
CA2140905 – 1994
EP0655910 – 1995
EP0655910 – 1996
JP8500033T – 1996
US5916554 – 1999
WO9403154 – 1994

Hospital Appointments:

Assistant Surgeon 1976-1983
Associate Surgeon - 1983-Present
Barnes Hospital, St. Louis, Missouri

Attending Surgeon 1982-1985
Consulting Surgeon 1976-1982
Associate Chief of Surgery 12/86-9/90
Veterans Administration Medical Center, St. Louis, Missouri

Consulting Surgeon 1985-7/95
Acting Chief of Surgery 3/86-12/86
St. Louis Regional Hospital, St. Louis, Missouri

Consulting Surgeon 1976-1985
St. Louis Children's Hospital, St. Louis, Missouri

Consulting Surgeon 1976-1985
Chief of Surgery 1981-1982
St. Louis County Hospital, St. Louis, Missouri

Attending Surgeon 1976-1982
St. Louis City Hospital, St. Louis, Missouri

Licensure: Missouri 1970

Certification: American Board of Surgery 1979
Fellow American College of Surgery 1982
Recertification: American Board of Surgery 1989

Medical Societies:

American College of Surgeons
American Diabetes Association
American Federation of Clinical Research
American Pancreatic Association
American Society for Artificial Internal Organs
American Surgical Association
Association for Academic Surgery
Society of University Surgeons
Tissue Culture Association
American Society of Transplant Surgeons
The Cell Transplantation Society
United Network of Organ Sharing - Region 8
International Pancreas & Islet Transplantation Association

Honors and Awards:

St. Louis Surgical Society Award for Research
Recipient 1973 & 1974
NIH Research Career Development Award
Recipient 1977-1982
NIH NAIMMDD Site Visit Teams
Member 1980-1995

NIH Surgery, Anesthesiology, and Trauma Study Section
Member 1985-1989
Reserve Member Status 1989-1995
World Journal of Surgery
Guest Editor - Islet Transplantation Symposium 1984
"Separation of Islet Cells in Microgravity by Continuous-Flow Electrophoresis", NASA - McDonnell Douglas Astronautics Corp. - STS-8, Space Shuttle, "Challenger", Experiment 1984

Editorial Reviewer:
Diabetes, Surgery, Journal Clinical Investigation
Grant Reviewer
Canadian Diabetes Association
Medical Research Council of Canada
National Surgical Advisor - Digestive Disease Center of Excellence - The Humana Corporation 1986-1994
Alpha Omega Alpha - Washington University Chapter - Elected Faculty Member January 1988
Outstanding Profession/Scientific Employee - Federal Employee of the Year Award Program - St. Louis Federal Executive Board 1990
The Huddinge Hospital Transplant Lectureship - Annual Meeting of the Swedish Society for Medical Science, Stockholm, Sweden, December 1990
Council Member - Cell Transplantation Society 1992-Present
Council Member - International Pancreas & Islet Transplantation Association 1993-Present
Editorial Board
Cell Transplantation 1992-1993
Transplantation Science

Committee Appointments:
Washington University Animal Studies Committee
Chairman 1991-1994
Washington University Medical Center Alumni Association
Committee Member 1991-1994
International Juvenile Diabetes Research Foundation Medical Science Review Committee 1990-1993
UNOS Pancreas Subcommittee Member 1991-1995
American Society of Transplant Surgeons Program and Publications Committee 1989-1991
Academic Freedom and Tenure Hearing Committee
Member 1985-1991
Washington University Committee on the Humane Care of Laboratory Animal Member
Member 1984-1990

Operating Room Technician Program
Forest Park Community College
Advisory Committee 1976-1995
Chairman 1986-1995
Mid-America Transplant Association
Member Professional Advisory Board 1985-1995
American Cancer Society Institutional Research Grants
Washington University Committee for Cancer Research
Member 1979-1989
Chairman 1982-1989
"Health Views" - Editorial Advisory Board
Member 1984-1988
American Diabetes Association, St. Louis Chapter
Research Committee 1985-1988
Department of Surgery Animal Facility
Director 1980-1984
Washington University Faculty Senate
Member 1981-1983
Executive Committee of the Faculty Council
Member 1982-1984
Clinical Sciences Research Building Animal Surgery Suite
Director 1984-1985
Department of Engineering Master Degree Thesis Review Committee:
1979-John Bergen - "Kinetics of Insulin Secretion from Pancreatic Islets of Langerhans and Development of Islet Transplantation Chambers"
1980-Paul Aegerter - "Microencapsulation of Living Cells to Prevent Immunological Response"
1983-Shiow Meei Lin - "Testing of a Mathematical Model for Islet Transplantation Chambers"
1987-Donna Wilkinson - "Coating of Live Cells with Polysaccharide Derivatives"
1989-Mary Blanchard - "Quantification of Low Concentrations of Polysaccharide Derivatives and Their Effect on Cell Viability"
1990-Ph.D. Thesis Review, Donna Hawk-Reinhard - "Purification of Pancreatic Islets of Langerhans Using Cell Electrophoresis"
St. Louis VAMC Committees
Comprehensive Planning Committee
Chairman 1988-1990
Administrative Executive Board 1988-1990
Professional Standards Board 1988-1990
Research Committee 1988-1994
District Planning Board 1988-1990

Barnes Hospital Committees
- Chaplaincy Committee 1992-1994
- Emergency Room Committee 1978-1984
- Search Committee for ER Director 1978-1984
- Patient Education Parent Committee 1979
- Surgery Patient Education Subcommittee Chairman 1981-1988

Tissue Culture Association
- Publicity Chairman 1980

Invited Presentations, Selected:

The Kroc Foundation
- Islet Transplantation Workshop 1974
- Islet Transplantation Workshop 1979
- Islet Transplantation Workshop 1982

National Institutes of Health
- National Conference on Diabetes 1979
- National Conference on Diabetes 1983

Juvenile Diabetes Foundation
- Conference on Research Tissue 1981
- National Meeting, Keynote Speaker 1984
- International Scientific Research Conference 1985

German Diabetes Association, Giessen, West Germany
- Islet Transplantation Workshop 1980
- Islet Transplantation Workshop 1989

American Society of Artificial Organs
- Annual Meeting - Keynote Speaker 1983
- Session Co-Chairman 1987

International Symposium on Organ Transplantation in Diabetes
- The Hague, Netherlands 1983

International Symposium on Kidney and Pancreas Transplantation
- Perugia, Italy 1984

International Islet Transplantation Workshop
- Canberra, Australia 1984

XII Congress of the International Diabetes Federation
- Madrid, Spain 1985

XIII Congress of the International Diabetes Federation
- Sydney, Australia 1989

National Disease Research Interchange
- Human Tissue Conference 1985
- Human Tissue Conference 1986
- Human Tissue Conference 1987
- Human Tissue Conference 1990

National Disease Research Interchange - Chairman of Task Force on "Biohazard and Contamination in the Use of Human Tissue and Organs"
Philadelphia, PA 1988
American Diabetes Association National Meeting - Session Co-Chairman for "Forms of Therapy" 1986
Visiting Scientist Program - University of Kansas Diabetes Center
Kansas City, Kansas 1986
Immunology of Diabetes Symposium - Member of International Advisory Committee
Edmonton, Canada 1986
International Symposium on Complications of Diabetes
The Hague, Netherlands 1986
Visiting Professorship - Department of Surgery - University of Minnesota
Minneapolis, Minnesota 1986
May 8th Endocrine Days
Victoria, British Columbia 1987
Second Annual Visiting Professorship in Diabetes - University of Wisconsin
Madison, Wisconsin 1987
First International Course on Transplantation
Venice, Italy 1987
Progress in Organ Transplants, Tissue Replacements and Implants
Sponsored by Biomedical Business International, New York 1987
Josiah Brown Memorial Symposium on Pancreas Beta Cell Transplantation
Los Angeles, California 1987
Seventh Workshop of the AIDSPIT Study Group
Igls, Austria 1988
First international Congress on Pancreatic and Islet Transplantation
Stockholm, Sweden 1987
Thirty-Fourth Annual Meeting of ASAIO, Invited Speaker "Modern Treatment of Insulin Dependent Diabetes"
Reno, Nevada 1988
Sixth Gordon Research Conference on Drug Carriers in Biology and Medicine
Plymouth, New Hampshire 1988
XII International Congress of the Transplantation Society
Sydney, Australia 1988
Second International Congress on Pancreatic and Islet Transplantation
Minneapolis, Minnesota 1989
Biology of Tissue Transplantation Symposium
Bethesda, Maryland 1989
Ninth Workshop of the AIDSPIT Study Group
Igls, Austria 1990
Society for Surgery of the Alimentary Tract Postgraduate Course, "Medical Aspects of Transplantation of the Liver, Pancreas and Intestine"
San Antonio, Texas 1990

Moderator for Pancreas Transplantation Scientific Session - American Society of Transplant Surgeons
Chicago 1990
UCLA Symposium on Molecular & Cellular Biology, "Tissue Engineering"
Keystone, Colorado 1990
The Huddinge Hospital Transplant Lectureship Annual Meeting of the Swedish Society for Medical Science
Stockholm, Sweden 1990
Third International Congress on Pancreatic and Islet Transplantation - Moderator and Plenary Speaker
Lyon, France 1991
European Association for the Study of Diabetes - Plenary Speaker
Dublin, Ireland 1991
Visiting Professor - University of Wisconsin
Madison, Wisconsin 1991
Moderator for Clinical Transplantation-Pancreas and Islets - XVIth International Congress of the Transplantation Society
Paris 1992
American Diabetes Association 53rd Annual Meeting - Plenary Speaker
Las Vegas, Nevada 1993
Fourth International Congress of Pancreas and Islet Transplantation - Plenary Speaker
Amsterdam 1993
IVth Joint Meeting of the Lawson Wilkins Pediatric Endocrine Society and the European Society for Pediatric Endocrinology - Symposium Speaker
San Francisco 1993
American Association for Clinical Chemistry
New York 1993

Publications

Abstracts:

1. Ballinger, W.F., Lacy, P.E., Scharp, D.W. Kemp, C.B., Knight, M. - Isografts and allografts of pancreatic islets in rats. Brit. J. Surg. 60:313, 1973

2. Kemp, C.B., Knight, M.J., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Islets of Langerhans injected into the portal vein of the diabetic rat. South African Journal of Surgery 11:135, 1973

3. Kemp, C.B., Knight, M.J., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Proceedings: Implantation of pancreatic islets into the portal vein of diabetic rats. Brit. J. Surg. 60:907, 1973

4. Scharp, D.W., Kemp, C.B., Knight, M.J., Murphy, J., Newton, W., Ballinger, W.F., Lacy, P.E. - Long term results of portal vein islet isografts and allografts in the treatment of Streptozotocin induced diabetes. Diabetes 23:359, 1974

5. Scharp, D.W., White, D.J., Ballinger, W.F., Lacy, P.E. - Transplantation of intact islets of Langerhans after tissue culture. In Vitro 9:364, 1974

6. Knight, M.J., Scharp, D.W., Kemp, C.B., Nunnelley, S.B., Ballinger, W.F., Lacy, P.E. - Cryopreservation of pancreatic islets. European Surgical Research 6(1):89, 1974

7. Ballinger, W.F., Murphy, J.J., Scharp, D.W., Hirshberg, G.E., Karl, R.C., Lacy, P.E. - Isolation and preservation of human islets of Langerhans for transplantation in the treatment of diabetes. European Society for Exp. Surg., Tenth Congress 1975

8. Griffith, R.C., Scharp, D.W., Ballinger, W.F., Lacy, P.E. - A morphologic study of intrahepatic portal vein islet isografts. Diabetes 34(2):419, 1975

9. Dodi, G., Scharp, D., Feldman, S., Maresca, B., Ballinger, W., Lacy, P. - Treatment of exocrine pancreatic dysfunction in diabetic rats by islet transplantation. European Surgical Research 9(1):98, 1977

10. Scharp, D.W., Merrell, R.C., Feldman, S., Ruwe, E., Feldmeier, M., Ballinger, W., Lacy, P. - Long term culture of islets of Langerhans utilizing a rotational culture method. In Vitro 13:174, 1977

11. Scharp, D., Krupin, T., Waltman, S., Oestrich, C., Feldman, S., Ballinger, W., Becker, B - Relationship of abnormal insulin release to fluorophotometry in experimental diabetes. Diabetes 27(2):435, 1978

12. Scharp, D.W., Merrell, R.C., Feldmeier, M.M., Downing, R., Ballinger, W.F. - Pseudo-islet formation and culture from canine isolated pancreatic cells. In Vitro 15:216, 1979

13. Rajotte, R.V., Scharp, D.W., Downing, R. Molnar, G.D., Ballinger, W.F. - The transplantation of frozen-thawed rate islets transported between centers, Diabetes 28:377, 1979

14. Downing, R. Scharp, D.W., Grieder, M., Ballinger, W.F. - Mass isolation of islets of Langerhans from the dog pancreas. Diabetes 28:426, 1979

15. Feldman, S.D., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Fetal pancreas isografts, cultured and uncultured to reverse Streptozotocin induced diabetes mellitus. The Association for Academic Surgery 12:116, 1979

16. Grieder, M.H., DeSchryver-Kecskemeti, K. Gingerich, R.L., Scharp, D.W. - In vitro studies using canine pseudo-islets and rat antrum cultures as models. UCLA Symposium, December 3, 1979

17. Scharp, D.W., Feldmeier, M.M., Rajotte, R.V., DeSchryver, K., Bell, M. - Human pseudo-islet formation, culture and preservation. Diabetes 29(2):18A, 1980

18. Gingerich, R.L., Scharp, D.W., Grieder, M.H., Dye, E.S., Mousel, K.A. - A new in vitro model to study secretion and biochemistry of pancreatic polypeptide (PP). Diabetes 29(2):30A, 1980

19. Bergen, J.F., Mason, N.S., Scharp, D.W., Sparks, R.E. - Insulin inhibition of islets in transplantation chambers. Presented at International Society for Artificial Organs Meetings, Paris, July 8-10, 1981

20. Sparks, R.E., Mason, N.S., Finley, T.C., Scharp, D.W. - Development, testing and modeling of an islet transplantation chamber, ASAIO Meetings, Chicago, April, 1982

21. Long, J.A., Adair, W.S., Scharp, D.W. - Hybridoma production against pancreatic cells. Diabetes 31(2):20A, 1982

22. Scharp, D.W., Hirshberg, G., Long, J.A. - The effect of islet dosage and time on rat portal vein isografts. Diabetes 31(2):162A, 1982

23. Scharp, D.W., Lacy, P.E. - The isolation and alteration of islet tissue for transplantation. The Tissue Culture Association Meeting, San Diego, June, 1982. In Vitro Suppl. 1, 1982

24. Long, J.A., Adair, W.S., Scharp, D.W. - An immunological approach to islet cell purification. J. Cell Biol. 95:4061, 1982

25. Sparks, R.E. Mason, N.S., Finley, T.C., Scharp, D.W. - Design of islet transplantation chambers giving a normal glucose tolerance test. ISAO Meetings, Kyoto, Japan, November, 1983

26. Sparks, R.E., Mason, N.E., Finley, T.C., Scharp, D.W. - Islet transplantation chamber models - assumption for insulin generation and glucose diffusion. International Symposium on Organ Transplantation in Diabetes, The Netherlands, September, 1983

27. Sparks, R.E., Mason, N.S., Scharp, D.W. - Some present directions in research on tissue transplantation chambers. International Conference on Artificial Organs, Glasgow, Scotland, September, 1983

28. Sparks, R.E., Mason, N.S., Finley, T.C., Scharp, D.W. - "A distributed source-model for hybrid artificial pancreas", presented by ASAIO, Toronto, Ontario, Canada, April, 1983

29. Scharp, D.W., Feldmeier, M.M., Olack, B.J., Swanson, C.J., O'Shaughnessey, S.F. - Electrophoretic purification of islet cells for transplantation. Diabetes 33(1):179A, 1984

30. Scharp, D.W., Rajotte, R.V., Kneteman, N.M., Lacy P.E. - Zero gravity electrophoresis of islet cells. 10th International Congress of the Transplantation Society Meeting, Minneapolis, August, 1984

31. Scharp, D.W., Lacy, P.E. - Human islet isolation and transplantation. Diabetes 34(1):5A, 1985

32. Kneteman, N.M., Alderson, D., Scharp, D.W. - Canine pancreatic islet allotransplantation: dose adjusted cyclosporine A vs azathioprine - steroid, Diabetes 34(1):62A, 1986

33. Alderson, D., Kneteman, N.M., Scharp, D.W. - The isolation of purified human islets of Langerhans. Diabetes 34(1):81A, 1986

34. Corlett, M.P., Fonseca, P., Scharp, D.W. - Detrimental effect of warm ischemia on islet isolation in rats and dogs with protection by oxygen free radical scavengers. Diabetes 36(1):223A, 1987

35. Scharp, D.W., Lacy, P.E., Finke, E.H., Olack, B.J. - Seven day culture of Ficoll purified human islets. Diabetes 36(1):222A, 1987

36. Corlett, M.P., Scharp, D.W. - Effect of warm ischemia on islet isolation in rats and dogs. The Association for Academic Surgery, Orlando, Florida, November 1-4, 1987

37. Misler, S., Gee, W., Scharp, D., Manchester, J., Falke, L. - Metabolically regulated potassium channels in human islet cells. Diabetes 37(1):6A, 1988

38. Atkinson, M.A., Maclaren, N.K., Riley, W.J., Scharp, D.W., and Holmes, L. - M_r 64,000 autoantibodies (64KA) predict insulin dependent diabetes (IDD). Diabetes 37(1):98A, 1988

39. Scharp, D.W., Lacy P.E., Ricordi, C., Boyle, P., Santiago, J.V., Gingerich, R.L., Anderson, C.B., Flye, M.W. - Human islet transplantation in patients with Type I diabetes. Presented at XII International Congress of the Transplantation Society, Sydney, Australia, August 14-19, 1988

40. Riley, W.J., Maclaren, N.K., Atkinson, M.A., Scharp, D.W., Lacy, P.E. - Islet autoantibodies in "prediabetes". Ninth International Workshop on the Immunology of Diabetes, Satellite Symposium on Beta Cell Destruction and Restoration in Type I Diabetes. November 27-29, Sydney, Australia, 1988

41. Atkinson, M.A., Maclaren, N.K., Riley, W.J., Scharp, D.W., Holmes, L. - The natural history and predictive value of M_r 64,000 islet cell antigen. Ninth International Workshop on the Immunology of Diabetes, Satellite Symposium of the International Diabetes Federation, Melbourne, Australia, 1988

42. Scharp, D.W., Chern, H.T., McCullough, C.S., Gingerich, R.L. - Lack of glucose induced insulin responsiveness in canine islets. 49th Annual Meeting of the American Diabetes Association, Detroit, Michigan, June 3-6, 1991. Diabetes 38(1):209A, 1989

43. Chern, H.T., Scharp, D.W., McCullough, C.S. - Cryogenic storage of purified canine islets. 49th Annual Meeting of the American Diabetes Association, Detroit, Michigan, June 3-6, 1989. Diabetes 38(1):246A, 1989

44. Callery, M.P., Ricordi, C., Scharp, D.W., Kamei, T., Lacy, P.E., Flye, M.W. - Hepatic insufficiency following portacaval shunting is prevented by prior intraportal pancreatic islet autotransplantation. The Society of University Surgeons, Baltimore, Maryland, 1989

45. Scharp, D.W., Corlett, M.P., Ricordi, C., Lacy, P.E. - Microbial contamination risk in human islet isolation. Study Group of the European Association for the study of Diabetes, Igls, Austria, 1989

46. Ricordi, C., Lacy, P.E., Socci, C., Finke, E.H. Dye, E., Olack, B.J., Swanson, C., Scharp, D.W. - Pancreatic islet isolation in mammals: Developments of the automated method. Study Group of the European Association for the Study of Diabetes, Igls, Austria, 1989

47. Callery, M.P., Kamei, T., Flye, M.W., Scharp, D.W. - Intrahepatic islet autografts in dogs function despite completely diverting portacaval shunt (PCS). Second International Congress on Pancreatic and Islet Transplantation, Minneapolis, Minnesota, 1989

48. McCullough, C., Chern, H.T., Scharp, D.W., Swanson, C.J., Olack, B.J., Davis, C.G. - Influence of transplant site on efficiency of canine islet cell auto-transplantation. Second International Congress on Pancreatic and Islet Transplantation, Minneapolis, Minnesota, 1989

49. Scharp, D.W. - islet Preservation - Short term for quality control testing. Presented at The Third International Conference on The Use of Human Cells, Tissues and Organs in Research. Washington, D.C., September 17-18, 1998

50. Falqui, L., Kowalski, L., Bittner, P., Olack, B.J., Scharp, D.W., Lacy, P.E. - Immunosuppressive regimens to prolong survival of islet xenografts transplanted across a wide species barrier (dog to mouse). Ninth Workshop of the AIDSPIT Study Group, Igls, Austria, January, 1990

51. Scharp, D.W., Lacy, P.E., Santiago, J.V., Boyle, P.J., Weide, L, Falqui, L., Marchetti, P, Gingerich, R.L., Cryer, P.E., Jaffe, A.S., McCullough, C.S., Anderson, C.B., Flye, M.W. - A clinical trial of islet transplantation. Ninth Workshop of the AIDSPIT Study Group, Igls, Austria, January, 1990

52. Marchetti, P., Falqui, L., Scharp, D.W., Lacy, P.E. - An improved method for large scale isolation of pure, viable islets of Langerhans from the adult porcine pancreas. Ninth Workshop of the AIDSPIT Study Group, Igls, Austria, January 1990

53. Scharp, D.W., Lacy, P.E., Santiago, J.V., McCullough, C.S., Weide, L.G., Falqui, L., Marchetti, P. Gingerich, R.L., Cryer, P.E., Jaffe, A.S., Anderson, C.B., Flye, M.W. - Preliminary results of clinical trials of human islet transplants. American Society of Transplant Surgeons 16th Annual Scientific Meeting, Chicago, Illinois, May 30-June 1, 1990

54. Marchetti, P., Scharp, D.W., Finke, E.H. - The isolation, function and xenotransplantation of porcine islets of Langerhans (PIL). 50th Annual Meeting of the American Diabetes Association, Atlanta, Georgia, June 16-19, 1990

55. Scharp, D.W., Lacy, P.E., McCullough, C.S., Weide, L., Boyle, P., Santiago, J.V., Gingerich, R., Cryer, P., Anderson, C.B., Flye, M.W. - Intraportal human islet transplants. XIII International Congress of The Transplantation Society, San Francisco, California, August 19-24, 1990

56. Finke, E.H., Marchetti, P., Swanson, C.J., Falqui, L., Lacy, P.E., Scharp, D.W. - Large scale isolation, function and transplantation of islets of Langerhans. XIII International Congress of the Transplantation Society, San Francisco, California, August 19-24, 1990

57. Olack, B.J., Swanson, C.J., McLear, M., Longwith, J., Scharp, D.W., Lacy, P.E. - Islet purification using Euroficoll gradients. XIII International Congress of the Transplantation Society, San Francisco, California, August 19-24, 1990

58. Scharp, D.W. - Clinical Trials. Presented at The Third International Conference on The Use of Human Cells, Tissues and Organs in Research. Washington, D.C., September 17-18, 1990

59. Swanson, C.J., Olack, B.J., Marchetti, P., Scharp, D.W. - Long-term metabolic and hormonal changes after intrasplenic canine islet transplantation. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

60. Olack, B.J., Marchetti, P., Swanson, C.J., Kneteman, N.M., Scharp, D.W. - No adverse effects of Cyclosporine A on either oral or intravenous glucose tolerance tests in Transplanted Dogs. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

61. McLear, M., Marchetti, P., Gingerich, R., Scharp, D.W. - The function of isolated human islets In Vitro. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

62. Marchetti, P., Swanson, C.J., Olack, B.J., McCullough, C.S., Scharp, D.W. - The effect of islet transplant site and mass on long-term metabolic function of canine islet autografts. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

63. Olack, B.J., Hill, A.C., Scharp, D.W., Lacy, P.E. - Automated purification of canine islets. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

64. McLear, M., Marchetti, P., Finke, E., Gerasimidi, A., Scharp, D.W., Lacy, P.E. - Porcine islet function: effects of arginine, leucine, and butyrate. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

65. Swanson, C.J., Marchetti, P., Newton, M., Scharp, D.W. - Effects of insulin therapy on glucose metabolism and insulin secretion in dogs and pancreatic islet autografts. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

66. Scharp, D.W., Lacy, P.E., Longwith, J.E. - Bacteriologic risks of human islet isolation for transplantation. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

67. Marchetti, P., Pfiffner, K., Scharp, D.W., Lacy, P.E., Gerasimidi, A., Finke, E.H. - Successful cryopreservation of porcine pancreatic islets (PI). Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

68. Marchetti, P., Swanson, C.J., Finke, E.H., Gerasimidi, A., Scharp, D.W., Lacy, P.E. - In Vitro function and xenotransplantation of long-term (3 weeks) cultured porcine islets of Langerhans. First international Congress on Xenotransplantation. Minneapolis, Minnesota, August 25-28, 1991

69. Marchetti, P., Giannarelli, Scharp, D.W., di Carlo, A., McLear, M., Lacy, P.E., Navalesi, R. - Metformin potentiates glucose stimulated insulin release: a study on diabetic patients and isolated human islets. European Association for the Study of Diabetes 27th Annual Meeting, Dublin, Ireland, September 10-14, 1991

70. Scharp, D.W., Marchetti, P., Swanson, C.J., Olack, B.J., Newton, M. - The effect of implantation site and islet mass on long-term survival and metabolic function of pure canine islet autografts. European Association for the Study of Diabetes 27th Annual Meeting, Dublin, Ireland, September 10-14, 1991

71. Marchetti, P., Finke, E., Pfiffner, K., Swanson, C., Gerasimidi-Vazeou, A., Olack, B., Scharp, D.W., Lacy, P.E. - Cryogenic storage of purified porcine islets. Presented at Study Group of the European Association for the Study of Diabetes, Igls, Austria, January 26-28, 1992

72. Scharp, D.W., Olack, B.J., Pfiffner, K., Gerasimidi-Vazeou, A., Finke, E.H., Lacy, P.E. - Preserving human islets prevents their rejection as xenografts. Presented at the First International Congress of the Cell Transplant Society, Pittsburgh, Pennsylvania, May 31-June 3, 1992

73. Marchetti, P., Scharp, D.W., Olack, B.J., Swanson, C.J., Bier, D., Cobelli, C., Toffolo, G., Newton, M., Navalesi, R. - Glucose metabolism, insulin sensitivity and glucagon secretion in dogs with intraportal or intrasplenic islet autografts. Presented at the First International Congress of the Cell Transplant Society, Pittsburgh, Pennsylvania, May 31-June 3, 1992

74. Marchetti, P., Scharp, D.W., Olack, B.J., Swanson, C.J. - Prediction of islet autograft failure by fasting glucagon:insulin ratio. Presented at the XIV International Congress of the Transplantation Society, Paris, France, August 16-21, 1992

75. Flavin, K.S., Scharp, D.W., Lacy, P.E. - Pre-islet transplant evaluation of psychiatric status using the National Institute of Mental Health (NIMH) Diagnostic Interview Schedule (DIS). Presented at the American Diabetes Association Meetings, San Antonio, Texas, June 20-23, 1992

76. Hawk-Reinhard, D.R., Scharp, D.W., Sparks, R.E. - Buffer solution temperature and velocity profiles in vertical micro-electrophoresis. Presented at American Institute of Chemical Engineers, New York, NY, November, 1992

77. Marchetti, P., Scharp, D.W., Finke, E., Pfiffner, K., Swanson, C., Gerasimidi-Vazeou, A., Olack, B., Navalesi, R., Lacy, P.E. - Insulin release upon perifusion and xenotransplantation of cryopreserved porcine islets. Presented at European Association for the Study of Diabetes, Igls, Austria, January 1992

78. di Carlo, A., Scharp, D.W., Gingerich, R.L., Ansara, M., Olack, B., Navalesi, R., Swanson, C. - Effect of culture and cryopreservation on insulin release from isolated, perifused human islets. Hormone and Metabolic Research 25:52, 1993

79. Marchetti, P., Scharp, D.W., Finke, E., Swanson, C.J., Gerasimidi-Vazeou, A., Olack, B.J., Navalesi, R., Lacy, P.E. - Prolongation of pig-to-mouse islet xenograft survival. Hormone and Metabolic Research 25:62, 1993

80. Hegre, O., Laurance, M., Harvey, J., Dionne, K., Appel, M., Scharp, D., Lacy, P.E. - Species variation in insulin secretory capacity of isolated islets. Hormone and Metabolic Research 25:58, 1993

81. Marchetti, P., Scharp, D.W., McLear, M., Finke, E.H., Olack, B.J., Swanson, C.J., Giannarelli, R. Navalesi, R., Lacy, P.E. - Pulsatile insulin secretion from isolated human pancreatic islets. Presented at the 53rd Annual Meeting of the American Diabetes Association Meeting, Las Vegas, Nevada, June, 1993

82. Shiroki, R., Poindexter, N., Mohanakumar, T., Scharp, D.W. - Rejection of human islet allografts in human lymphocyte reconstituted severe combined immunodeficient mice (Hu-Scid). Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

83. Marchetti, P., Scharp, D.W., Finke, E., Swanson, C.J., Gerasimidi-Vazeou, A., Olack, B.J., Navalesi, R., Lacy, P.E. - Effect of different temporary immunosuppressive therapies and low-temperature culture on pancreatic islet xenograft survival (pig-go-mouse). Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

84. Ansara, M.F., Saudek, F., Newton, M., Cryer, P.E., Scharp, D.W. - Islet transplantation prevents defective glucose counterregulation in diabetic dogs. Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

85. Scharp, D.W., Ansara, M.F., di Carlo, A., Flavin, K.S., White, N.H., Jendrisak, M.D., Lacy, P.E. - Intrasplenic islet transplants in kidney allograft recipients. Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

86. di Carlo, A., Scharp, D.W., Gingerich, R.L., Black, B.J., Swanson, C.J., Navalesi, R. - Insulin and glucagon release from isolated, perifused human islets following low temperature culture and cryopreservation. Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

87. Atkison, P., Zucker, P, Hramiak, I., Paul, T., McCallu, J., Stiller, KC.R., Scharp, D.W., Lacy, P.E., Olack, B., Finegood, D.T. - Continued insulin dependence despite normal insulin sensitivity and graft insulin secretion in a kidney/islet transplant patient. Presented at the Fourth International Congress on Pancreas and Islet Transplantation, Amsterdam, The Netherlands, June, 1993

88. Scharp, D.W., Swanson, C.J., Olack, B.J., Latta, P.P., Hegre, O.D., Gentile, F., Lacy, P.E. - Encapsulated human islet allografts in non-diabetic, Type 1 and Type 2 diabetic recipients. Presented at The 13th Workshop of the AIDSPIT Study Group, Igls, Austria, January 23-25, 1994. Horm. Metab. Res. 26:76, 1994

89. Scharp, D.W., Ansara, M.F., Brennan, D.C., White, N.H., Flavin, K.S., Zukosky, J.M., Cryer, P.E., Lacy, P.E. - Modification in methodology results in improvement in simultaneous kidney-islet success. Presented at the XVth World Congress of The Transplantation Society, Kyoto, Japan, August 28-September 2, 1994

90. Scharp, D.W., Brennan, D.C., White, N.H., Flavin, K.S., Lacy, P.E. - Increased islet mass raises rate of insulin independence in islet transplant recipients. Presented at IPITA 1995 Congress, Miami, Florida, June 18-22, 1995

Articles:

1. Merrick, A.W., Scharp, D.W. - Electroencephalography of resting behavior in cattle, with observations on the question of sleep, Am. J. Vet. Res., 32:1893-1897, 1971

2. Knight, M.J., Scharp, D.W., Kemp, C.B., Ballinger, W.F., Lacy, P.E. - Effects of cold storage on the function of isolated pancreatic islets, Cryobiology, 10:89-90, 1973

3. Kemp, C.B., Scharp, D.W., Knight, M.J., Ballinger, W.F., Lacy, P.E. - Importance of implantation site of pancreatic islet isografts in treatment of experimental diabetes, Surgical Forum 24:297-299, 1973

4. Kemp, C.B., Knight, M.J., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Transplantation of isolated pancreatic islets into the portal vein of diabetic rats, Nature 244:447, 1973

5. Scharp, D.W., Kemp, C.B., Knight, M.J., Ballinger, W.F., Lacy, P.E. - The use of Ficoll in the preparation of viable islets of Langerhans from the rat pancreas, Transplantation 16:686-689

6. Kemp, C.B., Knight, M.J., Scharp, D.W., Ballinger, W.F., Lacy, P.E. - Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats, Diabetologia 9:489-491, 1973

7. Scharp, D.W., Murphy, J.J., Newton, W.T., Ballinger, W.F., Lacy, P.E. - Transplantation of islets of Langerhans in diabetic Rhesus monkeys, Surgery 77:100-105, 1975

8. Scharp, D.W., Murphy, J.J., Newton, W.T., Ballinger, W.F., Lacy, P.E. - Application of an improved isolation technique for islet transplantation in primates and rats, Transplant. Proc. 6(1):739-741, 1975

9. Feldman, S., Hirshberg, G., Dodi, G., Raizman, M., Scharp, D., Ballinger, W., Lacy, P.E. - Intrasplenic islet isografts, Surgery 82:386-394, 1977

10. Karl, R.C., Scharp, D.W., Ballinger, W.F., Lacy, P.E. - Transplantation of insulin-secreting tissues, J British Society Gastroenterology - GUT 18:1062-1072, 1977

11. Griffith, R.C., Scharp, D.W., Hartman, B.K., Ballinger, W.F., Lacy, P.E. - A morphologic study of intrahepatic portal vein islet isografts, Diabetes 26:201-214, 1977

12. Feldman, S., Scharp, D., Hirshberg, G., Dodi, G., Ballinger, W., Lacy, P.E. - Streptozotocin-induced liver tumors, Transplantation 24:152-154, 1977

13. Feldman, S., Dodi, G., Haid, K., Scharp, D., Ballinger, W., Lacy, P.E. - Artificial hybrid pancreas, Surgical Forum 28:439-441, 1977

14. Scharp, D.W., Merrell, R.C., Feldman, S.D., Downing, R., Lacy P,E., Ballinger, W. - The use of gyrotational culture for the preservation of isolated islets, Surgical Forum 29:100-102, 1978

15. Krupin, T., Waltman, S.R., Scharp, D.W., Oestrich, G., Feldman, S.D., Becker, D., Ballinger, W.F., Lacy, P.E. - Ocular fluorophotometry in experimental diabetes mellitus: The effect of pancreatic islet isografts, Investigative Ophthalmology & Visual Science 18:1185-190, 1979

16. Downing, R., Scharp, D.W., Ballinger, W.F. - An improved technique for the isolation and identification of mammalian islets of Langerhans, Transplantation 29:79-83, 1980

17. Lacy, P.E., Davie, J.M., Finke, E.H., Scharp, D.W. - Prolongation of islet allograft survival, Transplantation 27:171-174, 1979

18. Scharp, D.W., Downing, R., Merrell, R.C., Grieder, M. - Isolating the elusive islet, Diabetes 29(1):19-30, 1980

19. Downing, R., Scharp, D.W., Merrell, R.C., Grieder, M., Ballinger, W.F. - Venous versus ductal distension of the dog pancreas for isolation of islets, Surgical Forum 30:422-424, 1979

20. Merrell, R.C., Scharp, D.W., Gingerich, R., Feldmeier, M.M., Grieder, M., Downing, R. - New approaches to the separation of islet cells, Surgical Forum 30:303-305, 1979

21. Feldman, S.D., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Fetal pancreas isografts, cultured and uncultured, to reverse streptozotocin induced diabetes mellitus, J. Surgical Research 29:309-318, 1980

22. Grieder, M.H., Gingerich, R.L., Scharp, D.W. - In Vitro studies of canine pseudo-islets. UCLA Symposium - Cellular Basis of Chemical Messengers in the Digestive System", Los Angeles, January 16-18, 1980

23. Silberberg, R., Scharp, D.W., Lesker, P.A. - Biochemical studies of bone from diabetic rats and rats bearing transplanted pancreatic islets, Experimental Cell Biology 49:61-67, 1981

24. Rajotte, R.V., Scharp, D.W., Downing, R., Preston, R., Molnar, G.D., Ballinger, W.F. - Pancreatic islet banking: the transplantation of frozen-thawed rat islets transported between Centers, Cryobiology 18:357-369, 1981

25. Britt, L.D., Scharp, D.W. - Formation of pseudo-islets from neonatal pig pancreas, Surgical Forum 31:354-356, 1980

26. Britt, L.D., Stojeba, P.C., Scharp, C.R., Grieder, M.H., Scharp, D.W. - Neonatal pig pseudo-islets: A product of selective aggregation, Diabetes 30:580-583, 1981

27. Britt, L.D., Scharp, D.W., Lacy, P.E., Slavin, S. - Transplantation of islet cells across major histocompatibility barriers after total lymphoid irradiation and infusion of allogeneic bone marrow cells, Diabetes 31:36-38, 1982

28. Bergen, J., Mason, N.S., Scharp, D.W., Sparks, R.E. - Insulin inhibition of islets in transplantation chambers, Artificial Organs 5:787-791, 1981

29. Paquette, T.L., Gingerich, R., Scharp, D.W. - Altered amidation of pancreatic polypeptide in culture, Biochemistry 20:7403-7408, 1981

30. Schmidt, R.E., Scharp, D.W. - Axonal dystrophy in experimental diabetic autonomic neuropathy, Diabetes 31:761-770, 1982

31. Yasunami, Y., Lacy, P.E., Scharp, D.W., Britt, L.D. - Isolation and transplantation of islet cells from the neonatal pig pancreas, Surgical Forum 33, 359-361, 1982

32. Gingerich, R.L., Scharp, D.W., Grieder, M.H., Dye, E.S., Mousel, K.A. - A new in vitro model for studies of pancreatic polypeptide secretion and biochemistry, Regulatory Peptides 5:13-25, 1982

33. Scharp, D.W., Long, J.A. - Studies of islet isolation and transplantation, Cryobiology 20:129-137, 1983

34. Long, J.A., Britt, L.D., Olack, B.J., Scharp, D.W. - Autotransplantation of isolated canine islets, Transplant Proc. 15:1332-1338, 1983

35. Schmidt, R.E., Santiago, B.P., Olack, B.J., Scharp, D.W. - The effect of pancreatic islet transplantation and insulin therapy on experimental diabetic autonomic neuropathy, Diabetes 32:532-540, 1983

36. Giddings, S.J., Rotwein, P., Chirgwin, J.M., Scharp, D.W., Permutt, M.A. - Analysis of insulin gene expression in human pancreas, Diabetes 32:777-780, 1983

37. Scharp, D.W. - Isolation and transplantation of islet tissue, World J. of Surgery 8:143-151, 1984

38. Scharp, D.W., Mason, N.S., Sparks, R.E. - Islet immunoisolation: the use of hybrid artificial organs to prevent islet tissue rejection, World J. of Surgery 8:221-229, 1984

39. Scharp, D.W. - Clinical feasibility of islet transplantation, Transplant Proc. 16(3):820-825, 1984

40. Heiken, J.P., Balfe, D.M., Picus, D., Scharp, D.W. - Radical pancreatectomy: Postoperative evaluation by CT, Radiology 153:211-215, 1984

41. Martin, K.W., Morian, J.P., Jr., Lee, J.K.T., Scharp, D.W. - Demonstration of splenic artery pseudoaneurysm by Magnetic Resonance Imaging, J. of Computer Assisted Tomography 9(1):190-192, 1985

42. Downing, R., Morrissey, S., Kiske, D., Scharp, D.W. - Does the purity of intraportal islet isografts affect their endocrine function?, J. Surg. Research 41(1):41-46, 1986

43. Scharp, D.W., Lacy, P.E., Alderson, D., Kneteman, N.M. - The procurement and distribution of cadaver human pancreas for islet isolation and transplantation. The Use of Human Tissues and Organs for Research and Transplant: Proceedings of the Second International Conference: National Diabetes Research Interchange April 1986:61-70

44. Scharp, D.W., Alderson, D., Kneteman, N.M. - Optimization of islet preparations for clinical transplantation. Transplant Proc. 18(6):1814-1816, 1986

59. Ricordi, C., Scharp, D.W., Lacy, P.E. - Mass isolation of human pancreatic islets for transplantation. Proceedings from XXVI World Congress of the International College of Surgeons, Milan, Italy, July 3-9, 1988, Monduzzi Editore S.p.A.

60. Corlett, M.P., Scharp, D.W. - The effect of pancreatic warm ischemia on islet isolation in rats and dogs, J. Surg. Research, 45:531-536, 1988

61. Ricordi, C., Kneteman, N.M., Scharp, D.W., Lacy, P.E. - Transplantation of cryopreserved human pancreatic islets into diabetic nude mice, World J. Surgery 12:861-865, 1988

62. Misler, S., Gee, W.M., Gillis, K.D., Scharp, D.W., Falke, L.C. - A metabolite-regulated ATP-sensitive K+ channel in human pancreatic islet cells, Diabetes 38:422-427, 1989

63. Scharp, D.W., Lacy, P.E., Ricordi, C., Boyle, P., Santiago, J., Cryer, P., Gingerich, R., Jaffe, A., Anderson, C.B., Flye, M.W. - Human islet transplantation in patients with Type 1 diabetes, Transplant Proc 21 (No. 1):2744-2745, February 1989

64. Scharp, D.W. - Islet Transplantation, Diabetes 38: (Suppl. 1):323-325, 1989

65. Ricordi, C., Lacy, P.E., Scharp, D.W. - Automated islet isolation from human pancreas, Diabetes 38 (Suppl. 1):140-142, 1989

66. Kneteman, N.M., Alderson, D., Scharp, D.W., Lacy, P.E. - Prolonged cyropreservation of purified human pancreatic islets, Diabetes 38:176-178, 1989

67. Kneteman, N.M., Alderson, D., Scharp, D.W., Lacy, P.E. - Long-term cryogenic storage of purified adult human islets of Langerhans, Diabetes 38:386-396, 1989

68. Brelje, T.C., Scharp, D.W., Sorenson, R.L. - Three-dimensional imaging of intact, isolated islets of Langerhans using Confocal microscopy, Diabetes 38:808-814, 1989

69. Schmidt, R.E., Plurad, S.B., Olack, B.J., Scharp, D.W. - The effect of pancreatic islet transplantation and insulin therapy on neuroaxonal dystrophy in sympathetic autonomic ganglia of chronic Streptozotocin diabetic rats, Brian Research 497:393-398, 1989

70. Callery, M.P., Ricordi, C., Scharp, D.W., Kamei, T., Swanson, C.J., Lacy, P.E., Flye, M.W. - Hepatic insufficiency following portacaval shunting is prevented by prior intraportal pancreatic islet autotransplantation. Surgery 106:257-266, 1989

71. Dotta, F., Colman, P.G., Nayak, R.C., Lombardi, D., Scharp, D.W., Andreani, D., Pontieri, G.M., di Mario, U., Lenti, L., Eisenbarth, G.S. - Ganglioside expression in human pancreatic islets, Diabetes 38:1478-1483, 1989

72. Permutt, M.A., Koranyi, L., Keller, K., Lacy, P.E., Scharp, D.W., Mueckler, M. - Cloning and functional expression of a human pancreatic islet glucose-transporter cDNA, Proc Natl Acad Sci USA 86:8688-8692, 1989

73. Callery, M.P., Kamei, T., Flye, M.W., Davis, C.G., Swanson, C.J., Olack, B.J., Lacy, P.E., Scharp, D.W. - Intrahepatic islet autografts in dogs function despite completely diverting portacaval shunt, Transplant Proc 22:767-770, 190

74. Scharp, D.W., Lacy, P.E., Santiago, J.V., McCullough, C.S., Weide, L.G., Falqui, L., Marchetti, P., Gingerich, R.L., Jaffe, A.S., Cryer, P.E., Anderson, C.B., Flye, M.W. - Insulin independence after islet transplantation into Type 1 diabetic patient, Diabetes 39:515-518, 1990

75. Atkinson, M.A., Maclaren, N.K., Riley, W.J., Scharp, D.W., Lacy, P.E. - 64,000 M_r autoantibodies as predictors of insulin dependent diabetes, Lancet 335:1357-1360, 1990

76. Scharp, D.W., Olack, B.J., Swanson, C.J., Marchetti, P. - Normal reproductive function in dogs with islet autograft, Transplant Proc 22(5):2403-2404, 1990

77. Chern, H.T., Scharp, D.W. - Viability assessment of cryopreserved canine islets in vivo, Endocrine Surgery 7(3):355-360, 1990

78. Medoff, G., Sagal, S., Krogstad, D., Peters, M., Hogan, J., Scharp, D. - Clinical Pathology Conference - Abdominal pain, fever, and weight loss in a 61 year-old Arabian Man, Am J Medicine 89:83-90, 1990

79. Ricordi, C., Gray, D.W.R., Hering, B.J., et al - Islet isolation assessment in man and large animals, Acta Diabetoal. Lat. 27:185-195, 1990

80. Atkinson, M.A., Maclaren, N.K., Holmes, L., Scharp, D.W., Lacy, P.E., Riley, W.J. - The natural history and predictive value of M_r 64,000 autoantibodies for insulin-dependent diabetes mellitus, J Autoimmunity 3:41-45, 1990

81. Atkinson, M.A., Holmes, L.H., Scharp, D.W., Lacy, P.E., Maclaren, N.K. - No evidence for serological autoimmunity to islet cell heat shock proteins in insulin dependent diabetes, J Clin Invest 87:721-724, 1991

82. Scharp, D.W., Lacy, P.E., Santiago, J.V., McCullough, C.S., Weide, L.G., Boyle, P.J., Falqui, L., Marchetti, P., Ricordi, C., Gingerich, R.L., Jaffe, A.S., Cryer, P.E., Hanto, D.W., Anderson, C.B., Flye, M.W., - Results of our first nine intraportal islet allografts in Type 1, insulin-dependent diabetic patients, Transplantation 51:76-85, 1991

83. Finke, E., Marchetti, P., Falqui, L., Swanson, C., McLear, M., Olack, B., Scharp, D.W., Lacy, P.E. - Large scale isolation, function and transplantation of islets of Langerhans from the adult pig pancreas, Transplant Proc 23(1):772-773, 1991

84. Olack, B., Swanson, C., McLear, M., Longwith, J., Scharp, D.W., Lacy, P.E. - Islet purification using Euroficoll gradients, Transplant Proc 23(1):774-776, 1991

85. Scharp, D.W., Lacy, P.E., Weide, L.G., Marchetti, P., McCullough, C.S., Flavin, K., Santiago, J.V. - Intraportal islet allografts: the use of a simulation index to represent functional results, Transplant Proc 23(1):796-798, 1991

86. Falqui, L., Finke, E.H., Carel, J.C., Scharp, D.W., Lacy, P.E. - Marked prolongation of human islet xenograft survival (human to mouse) by low-temperature culture and temporary immunosuppression with human and mouse anti-lymphocyte sera, Transplantation 51:1322-1324, 1991

87. Scharp, D.W., Lacy P.E. - The clinical feasibility of human islet transplantation, Clinical Diabetes 42-45, May/June, 1991

88. Marchetti, P., Finke, E.H., Gerasimidi-Vazeou, A., Falqui, L., Scharp, D.W., Lacy P.E.- Automated large scale isolation, *in vitro* function and xenotransplantation of porcine islets of Langerhans, Transplantation 52:209-213, 1991

89. Kowalski, L., Falqui, L., Lacy, P.E., Scharp, D.W. - Production of marked prolongation of survival of canine islet xenografts in mice by antilymphocyte sera and L3T4 antibody, Transplantation 52:1094-1097, 1991

90. Marchetti, P., Swanson, C., McLear, M., Finke, E.H., Gerasimidi-Vazeou, A., Scharp, D.W., Lacy, P.E. - In vitro function and xenotransplantation of long-term (3 weeks) cultured porcine islets of Langerhans, Transplant Proc 24(2):637, 1992

91. Kneteman, N.M., Marchetti, P., Tordjman, K., Bier, D.M., Santiago, J.V., Swanson, C.J., Olack, B.J., Scharp, D.W. - Effect of cyclosporine on insulin secretion and insulin sensitivity in dogs with intrasplenic islet autotransplants, Surgery 111(4):430-437, 1992

92. Scharp, D.W., Marchetti, P., Swanson, C.J., Newton, M., McCullough, C.S., Olack, B.J. - The effect of transplantation site and islet mass on long-term survival and metabolic and hormonal function of canine purified islet autografts, Cell Transplantation 1:245-254, 1992

93. Misler, S. Barnett, D.W., Pressel, D.M., Gillis, K.D., Scharp, D.W., Falke, L.C. - Stimulus-secretion coupling in B cells of transplantable human islets of Langerhans: evidence for a critical role for Ca^{2+} entry, Diabetes 41:662-670, 1992

94. Marchetti, P., Scharp, D.W., Longwith, J.E., Swanson, C.J., Olack, B.J., Gerasimidi-Vazeou, A., Finke, E.H., Lacy, P.E. - Prevention of contamination of isolated porcine islets of Langerhans, Transplantation 53(6):1364-1366, 1992

95. Olack, B.J., Hill, A., Scharp, D.W., Lacy, P.E. - Automated purification of canine islets, Transplant Proc 24(3):1003-1004, 1992

96. Marchetti, P., Pfiffner, K., Scharp, D.W., Lacy, P.E., Gerasimidi-Vazeou, A., Finke, E.H. - Successful cryopreservation of porcine pancreatic islets, Transplant Proc 24(3):1005-1006, 1992

97. Scharp, D.W., Lacy, P.E., McLear, M., Longwith, J.E., Olack, B.J. - The bioburden of 590 consecutive human pancreata for islet transplant research, Transplant Proc 24(3):974-975, 1992

98. McLear, M., Marchetti, P., Finke, E., Gerasimidi-Vazeou, A., Scharp, D.W., Lacy, P.E. - Porcine islet function: Effects of arginine, Leucine, and Butyrate, Transplant Proc 24(3):980-982, 1992

99. Marchetti, P., Finke, E.H., Swanson, C., Gerasimidi-Vazeou, A., Olack, B., Scharp, D.W., Navalesi, R., Lacy, P.E. - The potential of porcine islet xenotransplantation in the therapy of diabetes, Diab. Nutr. Metab. 5 (Suppl 1):151-154, 1992

100. Marchetti, P., Scharp, D.W., Olack, B.J., Swanson, C.J., Bier, D., Colelli, C., Toffolo, G., Newton, M., Navalesi, R. - Glucose metabolism, insulin sensitivity and glucagon secretion in dogs with intraportal or intrasplenic islet autografts, Transplant Proc 24(6):2828-2829, 1992

101. Marchetti, P., Olack, B., Swanson, C., Newton, M., Scharp, D.W. - Assessment of long-term (1 year) graft survival and metabolic hormonal changes after intrasplenic canine pancreatic microfragment transplantation, Diabetes & Metabolism 19:17-24, 1993

102. Barnett, D.W., Pressel, D.M., Chern, H.T., Scharp, D.W., Misler, S. - cAMP-Enhancing agents "permit" stimulus-secretion coupling in canine pancreatic islet ß-cells, J. Membrane Biol 138:113-120, 1994

103. Ansara, M.F., Saudek, F., Newton, M., Cryer, P.E., Scharp, D.W. - Pancreatic islet transplantation prevents defective glucose counter-regulation in diabetic dogs, Transplant Proc. 26(2):664-665, 1994

104. Shiroki, R., Poindexter, N., Mohanakumar, T., Scharp, D.W. - Rejection of human islet allografts in human lymphocyte-reconstituted severe combined immunodeficient mice, Transplant Proc. 26(2):716-717, 1994

105. Marchetti, P., Scharp, D.W., Finke, E., Swanson, C.J., Gerasimidi-Vazeou, A., Olack, B.J., Navalesi, R., Lacy, P.E. - Effect of different temporary immunosuppressive therapies and low-temperature culture on pancreatic islet xenograft survival (Pig-to-Mouse), Transplant Proc. 26(2):760-761, 1994

106. di Carlo, A., Scharp, D.W., Gingerich, R.L., Giannarelli, R., Ansara, M., Olack, B.J., Swanson, C.J., Navalesi, R. - Insulin and glucagon release from isolated, perifused human islets following low-temperature culture and cryopreservation, Transplant Proc. 26(2):821-822, 1994

107. Marchetti, P., Scharp, D.W., Pfiffner, K., Swanson, C.J., Finke, E.H., Olack, B.J., Gerasimidi-Vazeou, A., McLear, M., Lacy, P.E. - Cryogenic storage of isolated, purified porcine pancreatic islets, Transplantation 57:340-346, 1994

108. Marchetti, P., Kneteman, N.M., Swanson, C.J., Olack, B.J., Scharp, D.W. - Effect of cyclosporin treatment on metabolic and hormonal responses to mixed meal plus oral glucose in dogs with intrasplenic pancreatic islet autograft, Experimental Medicine, In Press

109. Marchetti, P., Scharp, D.W., McLear, M., Gingerich, R., Finke, E.H., Olack, B.J., Swanson, C.J., Giannarelli, R., Navalesi, R., Lacy, P.E. - Pulsatile insulin secretion from isolated human pancreatic islets, Diabetes 43:827-830, 1994

110. Shiroki, R., Poindexter, N.J., Woodle, E.S., Hussain, M.S., Mohanakumar, T., Scharp, D.W. - Human peripheral blood lymphocyte reconstituted severe combined immunodeficient (hu-PBL-SCID) mice: a model for human islet allograft rejection, Transplantation 58:1555-1562, 1994

111. Scharp, D.W., Swanson, C.J., Olack, B.J., Latta, P.P., Hegre, O.D., Doherty, E.J., Gentile, F.T., Flavin, K.S., Ansara, M.F., Lacy, P.E. - Protection of encapsulated human islets implanted without immunosuppression in patients with Type I and Type II diabetes and in non-diabetic controls, Diabetes 43:1167, 1994

112. Marchetti, P., Scharp, D.W., Finke, E.H., Giannarelli, R., Swanson, C.J., Olack, B.J., Lacy, P.E., Navalesi, R. - Factors affecting in vitro and in vivo function of isolated porcine islets, Transplant Proc. 26(6):3419-3420, 1994

113. di Carlo, A., Scharp, D.W., Gingerich, R.L., Ansara, M.F., Olack, B.J., Swanson, C.J., Giannarelli, R., Navalesi, R. - C-peptide secretion from isolated perifused human islets: effect of culture and cryopreservation, Transplant Proc. 26(6) 3399, 1994

114. Perdrizet, G.A., Rewinski, M.J., Schweizer, R.T., Scharp, D.W. - Heat shock and recovery protects pancreatic islets from warm ischemic injury, Transplant Proc. 26(6) 3477-3478, 1994

115. Marchetti, P., Scharp, D.W., Finke, E., Swanson, C.J., Gerasimidi-Vazeou, A., Olack, B.J., Navalesi, R., Lacy, P.E. - Anti-CD4 antibody treatment in xenografts of differently immunomodulated porcine islets, Transplant Proc. 26(3):1132, 1994

116. Brennan, D.C., Scharp, D.W., White, N.H., Flavin, K.S., Zukosky, J.M., Ansara, M.F., Cryer, P.E., Lacy, P.E. - Modification of methodology results in improvements in simultaneous kidney-islet success, Transplant Proc. 27(1):1349-1350, 1995

117. Chern, H.T., Scharp, D.W. - Successful long-term cryopreservation of highly purified canine islets, European Surg Res 27:167-175, 1995

118. Marchetti, P., Scharp, D.W., Finke, E.H., Swanson, C.J., Olack, B.J., Gerasimidi-Vazeou, A., Giannarelli, R., Navalesi, R., Lacy, P.E. - Prolonged survival of discordant porcine islet xenografts, Transplantation 61:1100-1102, 1996

119. Hill, R.S., Cruise, G.M., Hager, S.R., Lamberti, F.V., Yu, X., Garufis, C.L., Yu, Y., Mundwiler, K.E., Cole, J.F., Hubbell, J.A., Hegre, O.D., Scharp, D.W. - Immunoisolation of adult porcine islets for the treatment of diabetes mellitus, Annals of the NY Academy of Sciences 831:332-343, 1997

120. Cruise, G.M., Hegre, O.D., Scharp, D,W., Hubbell J.A., - A sensitivity study of the key parameters in the interfacial photopolymerization of poly(ethylene glycol) diacrylate upon porcine islets, Biotech. Bioeng. 57:655-665, 1998

121. Cruise, G.M., Scharp, D.W., Hubbell, J.A. - Characterization of permeability and network structure of interfacially photopolymerized poly(ethylene glycol) diacrylate hydrogels, Biomaterials 19:1287-1294, 1998

122. Cruise, G.M., Hegre, O.D., Lamberti, F.V., Hager, S.R., Hill, R., Scharp, D.W., Hubbell, J.A. - *In vitro* and *in vivo* performance of porcine islets encapsulated in interfacially photopolymerized poly(ethylene glycol) diacrylate membranes, Cell Transplantation, 8:293-306, 1999

<u>Letters, Reviews and Chapters</u>:

1. Karl, R., Scharp, D.W., Ballinger, W.F., Lacy, P.E. - Progress Report: transplantation of insulin secreting tissue, Gut. 18:1062-1072, 1977

2. Lacy, P.E., Scharp, D.W., Ballinger, W.F. - Transplantation of the islets of Langerhans - future directions. 14th International Congress of Therapeutics, p. 105, L'Expansion Scientique Francaise, Montpellier, France, 1977

3. Scharp, D.W. - Rejection of islet "isografts" in a strain of Lewis rats, Transplantation, 31:229-230, 1981

4. Rajotte, R.V., Scharp, D.W., Molnar, G.D., Preston, R. - Feasibility of low temperature banking for pancreatic islet cell transplantation. In "Islet Isolation, Culture, and Cryopreservation", Ed. K. Federlin and R.G. Bretzel. Thieme-Stratton, Inc., p. 124-135, 1981

5. Scharp, D.W., Downing, R., Merrell, R.C., Nunnelly, S., Kiske, D., Grieder, M.H. - New approaches in the methods for isolating mammalian islets of Langerhans utilizing the dog pancreas. In "Islet Isolation, Culture, and Cryopreservation". Ed. K. Federlin and R.G. Bretzel. Thieme-Stratton, Inc. p. 9-31, 1981

6. Slavin, S., Britt, L.D., Scharp, D.W., Lacy, P.E. - Treatment of experimental diabetes with histoincompatible islet allografts in rats conditioned with total lymphoid irradiation. In "Diabetic Renal-Retinal Syndrome", Volume 2. Ed. E.A. Friedman and E.A. L'Experance. Grune and Stratton, Inc., NY, p. 519-528, 1982

7. Chen, Y., Mason, N.S., Scharp, D.W., Ballinger, W.F., Sparks, R.E. - Collagenase immobilized on cellulose acetate membranes. In "Biomaterials: Interfacial Phenomena and Applications". Ed. S.L. Cooper and N.A. Peppas, American Chemical Society, Washington, D.C., p. 483-491, 1982

8. Scharp, D.W. - Hybrid Artificial Pancreas Panel Discussion. Transactions of the American Society for Artificial Internal Organs, 28:642-643, 1982

9. Scharp, D.W. - Clinical Feasibility of Islet Transplantation, International Symposium on Organ Transplantation in Diabetes, The Netherlands 1983, Transplant Proc. 16:820-825, 1984

10. Scharp, D.W., Mason, N.S., Sparks, R.E. - The use of hybrid artificial organs to provide immunoisolation to endocrine grafts. In "Bone Marrow & Organ Transplantation", Ed. S. Slavin, Elsevier Science Publishers, New York, p. 601-618, 1984

11. Scharp, D.W., Long, J.A., Feldmeier, M.M., Olack, B.J., O'Shaughnessy, S., Swanson, C.J. - Automated methods for isolating and purifying islet cells from the pancreas. In "Methods in Diabetes Research", Ed. Larner and Pohl, New York, John Wiley & Sons, p. 237-243, 1984

12. Scharp, D.W., Long, J.A., Feldmeier, M.M., Olack, B.J., O'Shaughnessy, S., Swanson, C.J. - Automated methods of mass islet isolation. In "Methods in Diabetes Research", Ed. Larner and Pohl, New York, John Wiley & Sons, p. 225-236, 1984

13. Slavin, S., Weiss, L., Weigensberg, M., Morecki, S., Fuks, Z., Britt, L., Lacy, P.E., Scharp, D.W. - Tollerance to alloantigens in rodents and reversal of streptozotocin-induced diabetes in tolerant rats by histo-incompatible islet allografts. In "Bone Marrow & Organ Transplantation", Ed. S. Slavin, Elsevier Science Publications, New York, 1984

14. Williamson, J.R., Olack, B.., Rowald, E.A., Chang, K., Scharp, D.W. - Effects of insulin treatment on collagen crosslinking in streptozotocin diabetic rats. In "Lessons from Animal Diabetes", Eds. E. Shafrir and A. Renold, John Libbey, London, 1984, p. 402-404

15. Lacy, P.E., Scharp, D.W. - Islet Transplantation in treating diabetes, Ann. Rev. Med,. 37:33-40, 1986

16. Scharp, D.W. - The elusive human islet: Variables involved in its effective recovery. In "Transplantation of the Endocrine Pancreas in Diabetes Mellitus", Eds. R. van Schifgaarde and M.A. Hardy, Elsevier Science Publications, Amsterdam, 1988, p. 97-107

17. Scharp, D.W., Lacy, P.E. - Islet transplantation: a review of the objective, the concepts, the problems, the progress and the future. In "Transplantation of the pancreas", Eds. J.M. Dubernard and D.E.R. Sutherland, Kluwer Academic Publishers, The Netherlands, 1989, p. 455-478

18. Scharp, D.W., Lacy, P.E. - Clinical islet transplantation. In "Excerpta Medica International Congress Series", Eds. R. Larkins, P. Zimmet, D. Chisholm, Elsevier Science Publishers, Amsterdam, 1989, p. 721-724

19. McCullough, C.S., Scharp, D.W., Lacy, P.E. - Human islet isolation and transplantation. In "Immunologische and Dyamische Aspekte der Insulinsubstitution beim Diabetes Mellitus", Ed. R.G. Bretzel, Springer-Verlag, Heidelberg, 1990, p. 260-267

20. Atkinson, M.A., Maclaren, N.K., Scharp, D.W. - No role for 65KD heatshock protein in diabetes, (Letter) Lancet 336:1250-1251, 1990

21. Lacy, P.E., Scharp, D.W. - Islet cell transplantation. In "Diabetes Mellitus", Fourth Edition, Eds. H. Rifkin and D. Porte, Jr., Elsevier Science Publications, New York, 1990, P. 880-886

22. McCullough, C.S., Scharp, D.W. - Pancreas and islet transplantation for the treatment of Type I diabetes mellitus. In "Surgical Management of the Diabetic Patient", Eds. M. Bergman and G.A. Sicard, Raven Press, Ltd., New York, 1991, p. 349-362

23. Scharp, D.W. - Islet Transplantation: Approaches to a Clinical Therapy. In "Perspectives in General Surgery", Vol 3, No. 1, Ed. Barry A. Levine, Quality Medical Publishers, St. Louis, MO, 1992, p. 93-105

24. Scharp, D.W. - Islet quality control testing and the islet isolation laboratory. In "Pancreatic Islet Cell Transplantation", Ed. Camillo Ricordi, R.G. Landes Company, Austin, 1992, p. 82-88

25. Scharp, D.W. - Clinical islet transplantation - registry report, accomplishments in the past and future research needs, Invited Commentary, Cell Transplantation 2:299, 1993

26. Scharp, D.W. - Pancreatic Islet Transplantation. In "Atlas of Organ Transplantation, Ed. M. Wayne Flye, W.B. Saunders Company, Philadelphia, PA, 1994, p. 209-220

27. Ansara, M.F., Cryer, P.E., Scharp, D.W. - Perioperative Management of the Diabetic Patient. In "Care of the Surgical Patient", Ed. A. McHugh, Scientific American Medicine, New York, NY, In Press

Non-Obese Diabetic (NOD) Mouse BAC Library

The Wellcome Trust Sanger Institute (Cambridge, United Kingdom) recently released to the scientific community a Non-Obese Diabetic (NOD) mouse BAC library containing 240,000 clones obtained from the Diabetes and Inflammation Laboratory (Cambridge, United Kingdom).

The NOD mouse, which spontaneously develops type 1 diabetes, is a valuable animal model that is used extensively in research exploring the etiology, prevention, and treatment of this disease. It is a vital research tool for testing promising prevention and treatment strategies at the preclinical level.

The Sanger Institute sequenced the complete NOD BAC library and used this resource to complete a physical map of the BAC clones. As a next step, they plan to sequence the 200,000 clones from the Pieter de Jong library (Children's Hospital, Oakland CA). These sequences will be aligned with those from the most recent version of the normal C57B1/6 (B6) mouse strain (a non-diabetic mouse strain) in an effort to identify single nucleotide differences between NOD mouse clone end sequences and the B6 mouse genome. Investigators at the Sanger Institute will use this sequencing information to identify and map candidate genes. Such information will guide efforts to isolate genes that contribute to the development of type 1 diabetes in humans.

This research was conducted as part of the Immune Tolerance Network, which is jointly funded by the National Institute of Allergy and Infectious Diseases, the National Institute of Diabetes, Digestive, and Kidney Disorders (both part of the National Institutes of Health) and the Juvenile Diabetes Research Foundation.

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◆ REVIEW ARTICLE ◆

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Development of new strategies to prevent type 1 diabetes: the role of animal models

Arno Hänninen[1], *Emma Hamilton-Williams*[2] *and Christian Kurts*[2,3]

Type 1 diabetes is an immune-mediated disease typically preceded by a long preclinical stage during which a growing number of islet-cell-specific autoantibodies appear in the serum. Although antigen-specific T lymphocytes and cytokines rather than these autoantibodies are the likely executors of β-cell-destruction, these autoantibodies reflect the existence of autoimmunity that targets islet β-cells. Abrogation of this autoimmunity during the preclinical stage would be the key to the prevention of type 1 diabetes. However, the quest of protecting islet-cells from the immune attack requires detailed knowledge of mechanisms that control islet-inflammation and β-cell-destruction, and of mechanisms that control immune tolerance to peripheral self-antigens in general. This knowledge can only be obtained through further innovative research in experimental animal models. In this review, we will first examine how research in non-obese diabetic mice has already led to promising new strategies of diabetes prevention now being tested in human clinical trials. Thereafter, we will discuss how recent advances in understanding the mechanisms that control immune response to peripheral self-antigens such as β-cell antigens may help to develop even more selective and effective strategies to prevent diabetes in the future.

Keywords: antigen-presentation; dendritic cells; disease prevention; immunological tolerance; non-obese diabetic mice; T-lymphocytes; transgenic animal models; type 1 diabetes

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Introduction

Type 1 diabetes (T1DM) is the clinical manifestation of the loss of insulin production in the endocrine pancreas. This is caused by immune-mediated destruction of islet β-cells. T1DM is believed to be an autoimmune disease, based on several lines of evidence (1–9). Although an infective (e.g., viral) or other environmental (e.g., dietary) agent may well be involved in the initiation of the immune attack towards islet β-cells (10, 11), it is clear that the (auto)immune attack itself is a key element in disease pathogenesis (12).

Immune responses against various islet-antigens appear even years before the clinical manifestation of T1DM, and precede the phase when sensitive metabolic tests first reveal attenuation in the function of β-cells (13). A number of islet-antigens are targeted by the immune system in T1DM, and cellular and/or humoral immune responses are detected against insulin, glutamic acid decarboxylase-65 (GAD 65), tyrosine phosphatase IA-2 and heat shock protein 60 (hsp 60) (14–16). Studies on pancreas samples obtained shortly after onset of clinical disease (as biopsies or as autopsy material following fatal ketoacidosis) have revealed the existence of cellular infiltrates in islets consisting of lymphocytes and antigen-presenting cells (APC), reflecting the immune attack and selective destruction of insulin-producing β-cells (4–6). Although studies in humans have been essential in the characterization of the disease process and its target antigens, development of novel therapeutic strategies requires studies also in experimental animal models.

The non-obese diabetic (NOD) mouse is the most widely used animal model of T1DM (17). This inbred mouse strain is unique in that it spontaneously develops autoimmune diabetes at high incidence. Although NOD mice harbour certain unique defects in their immune system (such as lack of the murine homolog of humal leukocyte antigen HLA-DR (18) and complement component C5 (19)) and have a

Abbreviations and acronyms

APC	antigen-presenting cell
CTLA-4	cytotoxic T-lymphocyte antigen-4
DC	dendritic cells
GAD65	glutamic acid decarboxylase-65
GM-CSF	granulocyte-macrophage colony-stimulating factor
HLA	human leukocyte antigen
IA-2	islet-associated-2 (tyrosine phosphatase, an autoantigen)
IL	interleukin
IFN	interferon
hsp 60	heat shock protein 60
LCMV	lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
NKT	natural killer cell-like T cell
NOD	non-obese diabetic mouse
OT-I T cell	OVA-specific CD8 T cell
OVA	ovalbumin
PD-L1	programmed death ligand 1
RANK	receptor for activation of NFκB
RIP	rat insulin promoter
TGF	transforming growth factor
T1DM	type 1 diabetes mellitus
TCR	T cell receptor
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
TRANCE	TNF-related activation induced cytokine
Treg	regulatory T cell

Key messages

- Type 1 diabetes (T1DM) is the clinical manifestation of immune-mediated destruction of insulin-producing β-cells which cannot be prevented yet.
- Most of the current clinical trials aiming at T1DM prevention are based on strategies developed in animal models.
- Research with these animal models has recently generated essential information regarding the cellular and molecular mechanisms regulating immune response to β-cells that can be used in the development of novel prevention strategies.

strong gender bias in diabetes incidence towards female preponderance (20), many of the important determinants are strikingly similar, including homologous susceptibility genes, major autoantigens, existence of insulitis and the ability of bone-marrow cells to transmit diabetes between individuals (21, 22).

In this review, we will first describe how research in non-obese diabetic mice has led to the discovery of new strategies of diabetes prevention that are now in human clinical trials. Thereafter, we will focus on work in transgenic animal models of islet-autoimmunity and discuss how immune tolerance towards sequestered self-antigens such as β-cell antigens is maintained, and how the tolerant state can change into a pathogenic immune response. Emphasis will be given to work elucidating the cellular and molecular basis of this balance and on therapeutic approaches that aim at posing this balance away from pathological autoimmunity.

The NOD mouse – a platform for testing existing immunomodulatory agents in the prevention of spontaneous autoimmune diabetes

Application of modern methods in molecular and cell biology to research on the mammalian immune system has allowed numerous receptor-ligand pairs and intracellular signalling pathways that regulate the function of lymphocytes and antigen-presenting cells to be identified. Through this research, an increasing number of antibodies and other reagents have become available for selective blockade or mimicking of the function of a specified molecule with a regulatory function. The NOD mouse is ideal for testing the effect on diabetes incidence of such agents. Thus, it forms a platform for identification and validation of potential drug targets and drugs in diabetes prevention.

In fact, surprisingly many different manoeuvres (over a hundred or so) have been reported to delay or diminish diabetes incidence in NOD mice (17). Many of these lack direct relevance for prevention of T1DM in humans simply because of their experimental nature. However, several potentially relevant approaches have been identified (Table 1 and references therein). As will be discussed later, some of the earliest human trials based on these approaches have now given negative outcomes that could reflect differences between T1DM in humans and the corresponding syndrome in NOD mice. However, results of other trials are more consistent with those obtained in NOD mice and support the idea that the NOD model can be used as a platform for testing various prevention protocols. Protocols applied in the NOD model have been based firstly on blocking the activation or function of T-lymphocytes or their subtypes and hence, of pathogenic effector cells, or their migration into pancreatic islets. Secondly, they have been based on administration of autoantigens in forms and schedules that are anticipated to enhance regulation of specific immune responses. Thirdly, numerous antigen-non-specific substances with immunomodulatory effects, or possible protective effect on β-cells have been applied with often favourable outcomes.

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It is conceivable that targeting a subtype of T-lymphocytes or a costimulatory pathway will affect the immune system also on a general level and induce various side effects. Therefore, there has long been a quest to develop strategies that selectively target the immune effector cells or locally inhibit tissue destruction. Accordingly, autoantigens (pro)insulin, GAD65 and hsp-60 or their peptides have been administered without immune adjuvants and/or *via* tolerogenic routes, especially intravenously or *via* mucosal surfaces (orally, intranasally or inhaled as aerosol). Results of these experiments have been promising (Table 1 and references therein) and have already led to human clinical trials, as detailed below. Unfortunately, initial results of these trials have been less encouraging (see below). According to animal experiments, oral and intranasal administration of antigen can also lead to induction of cytotoxic T-cell immunity (53–55) that may counteract its tolerance-promoting effects. This might need more attention in current trials where autoantigens are administered *via* mucosal routes. In animal models, a promising new strategy to induce regulatory immunity and protection from autoimmune diseases including diabetes is genetic (that is, DNA) vaccination with plasmids that encode an autoantigen (56). This strategy will be discussed later in this review.

Current clinical trials based on strategies developed in NOD mice

Strategies employed currently in clinical trials to prevent T1DM include the use of autoantigens in a tolerance-promoting form, targeting T-lymphocytes with antibody, protecting islet β-cells from the action of inflammatory cytokines and non-specific stimulation of regulatory cell types. Most of the ongoing trials, which are summarized in Table 2, are based on strategies developed in the NOD mouse. We will describe these trials shortly.

Anti-CD3 monoclonal antibody

hOKT3γ1[Ala-Ala] is a humanized non-activating anti-CD3 antibody engineered to lack Fc-Receptor binding domains (57). Preclinical studies in the NOD mouse showed that antibody treatment could effec-

Table 1. Examples of protocols that prevent diabetes in NOD mice and have a relevant target mechanism.

Protocol	Mechanism of action	Antigen specificity	Reference
Treatment with an antibody against:			
CD3	Depletion or inactivation of islet-infiltrating T cells/induction of regulatory immunity	no	(23, 24)
CD4/CD8	Depletion/inactivation of CD4 or CD8 T cells	no	(25)
TCR	Depletion/inactivation of T cells	no	(26)
CD86(B7.2)	Interference with T cell activation	no	(27)
CD45RB	Interference with T cell activation	no	(28)
CD40L	Interference with T cell activation	no	(29)
VLA-4 (α4-integrin)	Prevention of lymphocyte accumulation in islets and/or activation of diabetogenic T cells	no	(30, 31)
MAdCAM-1	Prevention of lymphocyte accumulation in islets and/or activation of diabetogenic T cells	no	(32)
ICAM-1/LFA-1	Prevention of lymphocyte accumulation in islets and/or activation of diabetogenic T cells	no	(33)
Treatment with:			
adjuvant (IFA)	immune deviation	no	(34)
vitamin D3 deriv.	immune deviation	no	(35)
αGal-ceramide	activation of regulatory NK T cells	no	(36)
interleukin 4	immune deviation/regulatory cells	no	(37)
interleukin-10	immune deviation/regulatory cells	no	(38, 39)
nicotinamide	protection of target β-cells against NO	no	(40)
subcutaneous insulin	Inactivation of pathogenic T cells	yes	(41, 42)
oral, intranasal or aerosol insulin	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(43–46)
insulin-encoding DNA-plasmid	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(47)
intranasal GAD peptides	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(48)
GAD-encoding DNA-plasmid	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(49, 50)
intrathymic GAD	Deletion of GAD-specific T cells from the pool of circulating mature T cells	yes	(51)
HSP 60	Inactivation of pathogenic T cells	yes	(52)
p277 of HSP 60	Inactivation of pathogenic T cells	yes	(52)

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Table 2. Currently ongoing clinical trials to prevent diabetes.

Trial	Principle/hypothesis	Evidence from NOD mice	Status	Results[a]
hOKT-3γ1	Inactivation of T cells. Induction of regulatory immunity	yes	Phase I-II	+
p277 of HSP-60	Induction of tolerance towards one autoantigen	yes	Phase I	+
insulin i.v. and s.c.[b]	Induction of tolerance towards one autoantigen	yes	Phase II	–
insulin s.c. in adjuvant (IFA)[c]	Induction of tolerance towards one autoantigen	yes	Phase II	N.A.
insulin *via* oral route	Induction of tolerance towards one autoantigen	yes	Phase II	N.A.
insulin *via* intranasal route	Induction of tolerance towards one autoantigen	yes	Phase III	N.A.
GAD 65 s.c.	Induction of tolerance towards one autoantigen	yes	Phase II	N.A.
nicotinamide *via* oral route	Protection of islet β-cells	yes	Phase III	–
IFN-α	Modulation of immune response	yes	Phase II	N.A.
Lactobacillus *via* oral route	Stimulation of regulatory NK-T cells	yes	Phase II	N.A.
Apl[d] of insulin B-chain	Induction of tolerance to insulin	yes	Phase II	N.A.
Vitamin D3 derivative	Modulation of immune response	yes	Phase II	N.A.
Exclusion of bovine proteins from diet in infancy	Avoidance of early immune response towards putative 'mimics' of autoantigens and/or sensitization of immature gut to foreign proteins	not directly	Phase III	N.A.

[a] Results: + positive, – negative results; N.A. results not available yet; [b] i.v. = intravenous administration; s.c. subcutaneous administration; [c] IFA = incomplete Freund's adjuvant; [d] *Apl* = altered peptide ligand.

tively 'cure' diabetic mice, restoring normoglycemia (24) or prevent disease development in pre-diabetic mice (58, 59). The antibody appears to bind to all CD3 expressing T-cells resulting in partial T cell receptor (TCR) signalling, which then has different outcomes depending on the cell type triggered. The overall outcome being killing or anergy induction in Th1 type cells (producing interleukin-2 or interferon-γ) and stimulation of Th2 type cells (cells producing interleukin-4 or interleukin-10) (60, 61). In an intervention trial, a 14 day course of intravenous hOKT3-γ1[Ala-Ala]) was used in recent onset (within 6 weeks) T1DM patients and followed for one year. Treatment resulted in sustained or improved C-peptide responses in 9 out of 12 treated patients compared with a sustained response in only 2 out of 12 control patients. Treated patients also needed significantly less insulin over the year following diagnosis. A transient depletion (36%) of lymphocytes followed antibody treatment, which later returned to normal levels. Responding patients had a decreased ratio of CD4:CD8 T cells after repopulation. Mild side effects included anti-idiotype antibodies, mild fever and an eczematous dermatitis-like rash. This trial is now being expanded to a multicentre phase II trial involving about 80 patients after these promising results. Further details are available at http://www.immunetolerance.org/research/autoimmune/trials/herold1.html

Hsp-60 peptide p277

One of the many self antigens, which are reacted against in T1DM, is heat shock protein 60. NOD mice contain autoreactive T cells specific for peptide 277 derived from this protein and immunisation of adult NOD mice with this peptide could prevent and occasionally revert diabetes (52). The mechanism of action is believed to be stimulation of Th2 type hsp60-reactive T cells resulting in a change in the cytokine milieu away from inflammatory cytokines (62). DiaPep277 is the human form of this peptide, which has been modified at two residues to increase stability *in vivo* (63). In a randomised double blind phase II trial DiaPep277 was injected subcutaneously in mannitol and vegetable oil at 0, 1 and 6 months following entry into the trial (63). Adult male patients were on average 12–15 weeks post-diagnosis and were followed for only 10 months. At the end of the study mean C-peptide levels had been maintained in treated patients whereas they had fallen in controls. Insulin requirements were also significantly lower at 10 months. Further phase II and phase III trials are now beginning based on these suggestive results.

Subcutaneous insulin therapy

Insulin has long been viewed as a primary target for tolerisation therapy in T1DM. It was shown that incidence of diabetes was significantly reduced in NOD mice given a low dose of prophylactic insulin from weaning until 180 days of age (41). Similar findings were also observed in the BioBreed rat, another animal model of T1DM (64). This phenomenon was believed to be due to 'beta-cell rest' in which there was a lower requirement for insulin secretion by the beta cells and less release of islet autoantigens associated with insulin secretion. These findings paved the way to the establishment of the Diabetes prevention trial (DPT-1) in which either subcutaneous or oral insulin was given to high risk relatives of T1DM patients. In the subcutaneous branch of the trial 84,228 relatives were assessed immunologically, metabolically and genetically for risk of disease

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development over 5 years (DPT-T1D study group 2002). A group of 169 high risk (>50% over 5 years) relatives then underwent intervention of daily s.c. insulin injections and 170 controls received no treatment. The incidence of diabetes after 5 years was identical in both groups (69 *versus* 70 diabetic subjects). The reason for the negative outcome in the DPT-1 trial compared with the positive results in animal models and a pilot trial in humans (65) is unknown. It may be due to the dosage protocol or the difference in time of intervention. In mice, therapy began at a young age before the development of insulitis whereas relatives were selected on the basis of showing signs of autoimmunity (presence of islet autoantibodies) already.

Mucosal insulin

The second part of the DPT-1 trial is ongoing and used the 'intermediate' risk (25%–50% risk of developing T1DM over 5 years) relatives of T1DM patients. Patients receive continuous oral insulin or placebo and are being followed for 6 years for diabetes development. This intervention was not predicted to prevent diabetes by 'beta-cell rest' as subcutaneous insulin was but rather through active tolerisation upon antigen uptake at a mucosal surface (66). In NOD mice, an active (transferable), form of tolerance can be induced by feeding mice insulin but it is highly dose-dependent (43, 44). Therefore it is likely that in transferring this therapy to the human situation the dosage protocol will be critical.

A second major trial, the diabetes prediction and prevention project (DIPP), also seeks to tolerise individuals predicted to have a high risk of progression to T1DM by mucosal insulin administration. In this study babies are screened at birth for genetic HLA markers predisposing them to T1DM and then undergo immunological follow up. At risk children are then enrolled in a prevention trial to test intranasal insulin *versus* placebo for its ability to prevent or delay disease onset over three years. Aerosol insulin and intranasal insulin peptide administration have both been shown to be efficacious in prevention of diabetes in the NOD model (45, 46), although intranasal antigen administration has also been shown to induce potent cytotoxic T-cell immunity (67). For further details, see http://www.utu.fi/research/dipp/engdexx.htm

Subcutaneous insulin B chain in IFA

Another trial also attempts to induce tolerance to the metabolically inactive B chain of insulin by subcutaneous injection in Incomplete Freunds Adjuvant (IFA). In the NOD mouse, subcutaneous insulin or insulin B-chain given in IFA generated a transferable suppressive effect (42). In this study patients with insulin autoantibodies receive one injection within one month of diagnosis of T1DM and will be followed for two years. For further details, see http://www.immunetolerance.org/research/autoimmune/trials/orban1.html

Subcutaneous GAD65 in alum

An important early autoantigen in diabetes in both the NOD mouse and humans is GAD65 (51). A phase II trial will test the ability of recombinant GAD65 in alum to halt disease progression in recent onset patients. The vaccine called Diamyd will be injected subcutaneously two times four weeks apart in a range of doses. For further details, see http://www.diamyd.com/docs/research.html

Nicotinamide

A different approach, which is not antigen specific, attempts to use high doses of the B-vitamin nicotinamide to prevent or delay T1DM onset. Nicotinamide acts on the islet beta cells themselves making them more resistant to autoimmune attack. Nicotinamide is thought to primarily target the enzyme poly(ADP-ribose)polymerase (PARP) which is upregulated early after exposure to nitric oxide or reactive oxygen intermediates. Such exposure causes depletion of intracellular nicotinamide adenine dinucleotide (NAD^+) and results in death of sensitive cell types such as beta cells (68). Early nicotinamide treatment of NOD mice prevented diabetes partially and reduced insulitis severity (40). The results of initial clinical trials (69–71) have been controversial, since, while Chase et al. reported no effect of oral administration of nicotinamide, both Mendola and collaborators and Pozzilli et al. observed an increased stimulated C-peptide secretion in postpubertal patients on nicotinamide.

Nicotinamide has also been used in prevention trials with relatives of T1DM patients. One German study of 25 nicotinamide treated and 30 placebo control subjects showed no effect on disease progression (72). However, a larger study in New Zealand involving schoolchildren who had islet-cell autoantibodes obtained results that suggested a protective effect (73). The European nicotinamide diabetes intervention trial (ENDIT) is a larger clinical trial involving 552 high-risk relatives taking daily oral nicotinamide or placebo. For further details and for the recently disclosed negative results of this trial, see http://www.bris.ac.uk/Depts/DivMed/endit.html

Interferon-α

One successful strategy to prevent disease in the NOD

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mouse involves altering the cytokine milieu to an anti-inflammatory, i.e., interleukin (IL)-4 and IL-10 containing environment. This has been done by injection of these cytokines, transgenic expression or by stimulation of cells which can produce them endogenously. Brod and co-workers have fed NOD mice with interferon-α, which suppressed diabetes and stimulated mitogen induced IL-4, IL-10 and interferon (IFN)-γ production in splenocytes (74). This was then trialled in 10 newly diagnosed T1DM patients resulting in preserved beta-cell function in 8 of these patients after 12 months (75). This study has now been expanded to a larger phase II trial involving 120 newly diagnosed patients taking oral IFN-α daily. It is unclear however what the exact mechanism of this therapy is, as interferon administered orally does not appear to be adsorbed into the bloodstream (76). Additionally, the presence of transgenically expressed IFN-α in the islet beta cells themselves actually precipitates diabetes (77). As such it is possible that interferon-α acts on lymphocytes in the gut, which then circulate to have a wider regulatory effect.

Oral administration of lactobacillus

Another study also attempts to modulate the immune response *via* the gut. Maclaren and co-workers are conducting a trial of oral lactobacillus in recently diagnosed T1DM patients. They propose that this will stimulate natural killer cell-like T (NKT) cell activity as administration of lactobacillus plantarum to children with HIV boosted this cell-subset (78). NKT cells are a regulatory-cell subset which have been reported to be deficient in T1DM patients (79, 80) although a more recent study utilising a more direct tetramer based method of identifying NKT cells refutes this claim (81). In the NOD mouse both transfer of this cell subset or injection of a glycolipid ligand to activate existing NKT cells can prevent diabetes (36, 82, 83). Lactobacillus casei feeding to NOD mice prevents disease (84) and various lactobacilli strains have been shown to be strong stimulators of IL-12 and IL-10 (85). However, strong evidence of lactobacillus stimulation of NKT cells has yet to be demonstrated.

Altered peptide ligand NBI-6024

An interesting antigen specific therapy involves the identification of peptides with similar sequences to immunodominant epitopes, which have modulatory activity. In the NOD mouse peptide 9-23 of the insulin B chain is recognised by a large proportion of pathogenic CD4 T-cells derived from the insulitic infiltrate (86–88). Alleva and colleagues searched for peptide analogues of $B_{(9-23)}$ which could inhibit $B_{(9-23)}$ specific T-cell responses and used one of these, NBI-6024, to test its therapeutic effect in the NOD mouse (89). Therapeutic altered peptide ligands are thought to function as competitive inhibitors of the native peptide by having a high binding affinity for MHC while concurrently engaging the TCR in a non-productive manner (90). They have also been shown to be effective in stimulating regulatory cell subsets, which can transfer protection between animals (91). The altered peptide ligand NBI-6024 is now being used in a large phase II study in which new onset T1DM patients will receive monthly injections of this medication at three different doses for two years.

1,25-Dihydroxy-Vitamin D3

The activated form of vitamin D3, 1,25-Dihydroxy-Vitamin D3, has been shown to act directly on the immune system *via* specific receptors on APCs and activated T cells (92). Its effects are immunosuppressive and include inhibition of IL-2 and IL-12 production and Th1-type responses (93). Importantly 1,25-Dihydroxy-Vitamin D3 has been shown to inhibit dendritic cell maturation, both *in vitro* and *in vivo* (94, 95). Administration of an analogue of 1,25-Dihydroxy-Vitamin D3 to NOD mice significantly reduced the incidence of diabetes (96) and enhanced the number of $CD4^+$ $CD25^+$ cells of regulatory phenotype in the pancreatic lymph node (97). 1,25-Dihydroxy-Vitamin D3 administered daily for 9 months in recently diagnosed T1DM patents is being trialled in a German study. For further details on this trial see http://www.roche.com/pages/downloads/science/pdf/rtdcmannh02-3.pdf

Withdrawal of cow's milk proteins from diet during infancy

According to one hypothesis (not directly derived from findings in NOD mice), exposure of the immature digestive tract in infancy to casein or other proteins in cow's milk formula or soy-based formula has a role in the early pathogenesis of type 1 diabetes. A casein-free diet has been tested in the NOD mouse after weaning and was shown to be effective in preventing diabetes (98). Although contradictory results were achieved from previous studies in humans evaluating whether introduction of formula during infancy is associated with the development of type 1 diabetes later in life (99, 100), a larger phase III clinical trial called TRIGR (Trial to Reduce IDDM in the Genetically at Risk) has been initiated to test if the hypothesis holds true or not. Infants that are determined to have a high risk of developing type 1 diabetes are eligible. After weaning from breast-feeding, they will receive hydrolyzed formula that does not contain intact proteins, or standard cow's milk-based formula. Infants will have at least a two-

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month exposure to the study formulae and then will be monitored for up to ten years. For further details, see http://www.trigr.org/about.html

Transgenic mouse models in the study of T cell-mediated autoimmunity against pancreatic islets

Investigations in humans with T1DM and studies performed in the NOD mouse model have clearly demonstrated the crucial role of autoreactive CD4 helper and CD8 effector T cells in the pathogenesis of autoimmune diabetes (101). In NOD mice, numerous autoantigens are recognized by these T cells (see above). The relevance of these antigens for disease initiation and progression, and the mechanisms of their processing and presentation, however, are still unclear. Consequently, techniques and tools other than the NOD model had to be developed. The most valuable models were generated by genetically introducing well characterized model antigens into islet-β-cells, under the assumption that such neoantigens will be handled like endogenous islet antigens by the immune system (102). Transgenic neoantigens, however, are often aberrantly expressed in the thymus, resulting in the deletion of endogenous transgene-specific T cells. This problem could be overcome by intravenous injection of specific T cells, which are usually obtained from congenic T cell receptor – transgenic mice. Since their antigen (the model autoantigen) is not expressed in the donor mice these T cells are not negatively selected in the thymus. Importantly, the absence of antigen during T cell development ensures a completely naïve phenotype of the autoreactive T cells. This is crucial because it allowed, for the first time, investigation of the activation of T cells specific for a pancreatic self antigen *in vivo* (103). The other major advantage of adoptive transfer experiments is the ability to label the T cells with fluorescent dyes prior to injection, which allows them to be easily tracked *in vivo*. This technique can also reveal their activation and proliferation through analysis of cell division (104, 105). These innovations allowed many important aspects of T cell-mediated autoimmune responses to be addressed, including the site of activation of the autoreactive T cells, the influence of their numbers, their fate and the type of antigen-presenting cell responsible. Although parameters such as T-cell affinity and epitope spreading and recruitment of other autoreactive T cell clones are not taken into account, this approach has added considerably to our knowledge of immune-mediated destruction of islet β-cells by allowing us to dissect the actions of relevant components and their interactions which finally result in disease (102).

The approach described above has been particularly successful in elucidating the role and physiology of CD8 T effector cells. As compared to CD4 T helper cells, CD8 T cells are difficult to maintain *in vitro* as T cell lines, mainly because of their cytotoxicity. They quickly destroy APCs *in vitro*, and thus deprive themselves of antigenic survival signals. Furthermore, the MHC class I molecules, which are recognized by CD8 T cells, are expressed by nearly all murine cells, including T cells, so that CD8 T cells could potentially kill each other *in vitro*. These problems were overcome by generating naïve, and therefore unarmed CD8 T cells in transgenic donor mice. Such T cells became cytotoxic only after transfer into recipient mice expressing antigen. Transgenic CD8 T cells specific for certain MHC class I molecules were first generated (106). These T cells were introduced into transgenic mice expressing MHC I molecules under the influence of the rat insulin promoter (RIP) in pancreatic islet cells. This resulted in immune tolerance, unless the T cells were supplied with inflammatory mediators (107). These experiments demonstrated that autoreactive CD8 T cells can in principle destroy β-cells and cause diabetes (102) although their specificity for antigenic peptide was unclear. Therefore, they did not allow investigation of the processing of islet antigens. This limitation was overcome in the next generation of CD8 T cell transgenic systems. The first of these models, the RIP-mOVA model, expressed ovalbumin (OVA) as a model antigen in pancreatic islet cells. OVA-derived peptides could be detected with transgenic OVA-specific CD8 and CD4 T cells derived from OT-I and OT-II mice, respectively (OT-I and OT-II refer to the transgenic OVA-specific T cells, called OT-I and OT-II cells, that are produced by these transgenic mice). These experimental systems demonstrated clearly that both naïve CD4 and CD8 T cells with specificity for a pancreatic self antigen are activated by dendritic cells in the pancreatic lymph nodes, and not in the islets (108) (Fig 1). The DCs presumably took up β-cell antigens in the islets and carried them to the pancreatic lymph node for presentation to naïve T cells. In the case of CD8 T cells, these results demonstrated so-called cross-presentation, which denotes the presentation of extracellular antigens with MHC I molecules to CD8 T cells.

Since then, numerous observations of cross-presentation and cross-priming *in vivo* have been described, not only of transgenic self-antigens in models like the RIP-mOVA or the hemagglutinin system (109), but also of tumour (110) and viral antigens (111, 112). Thus, the basic mechanisms of antigen presentation and T-cell activation *in vivo* that were uncovered in transgenic systems have been verified for many 'natural' antigens. This is not surprising, because indirect presentation of antigen

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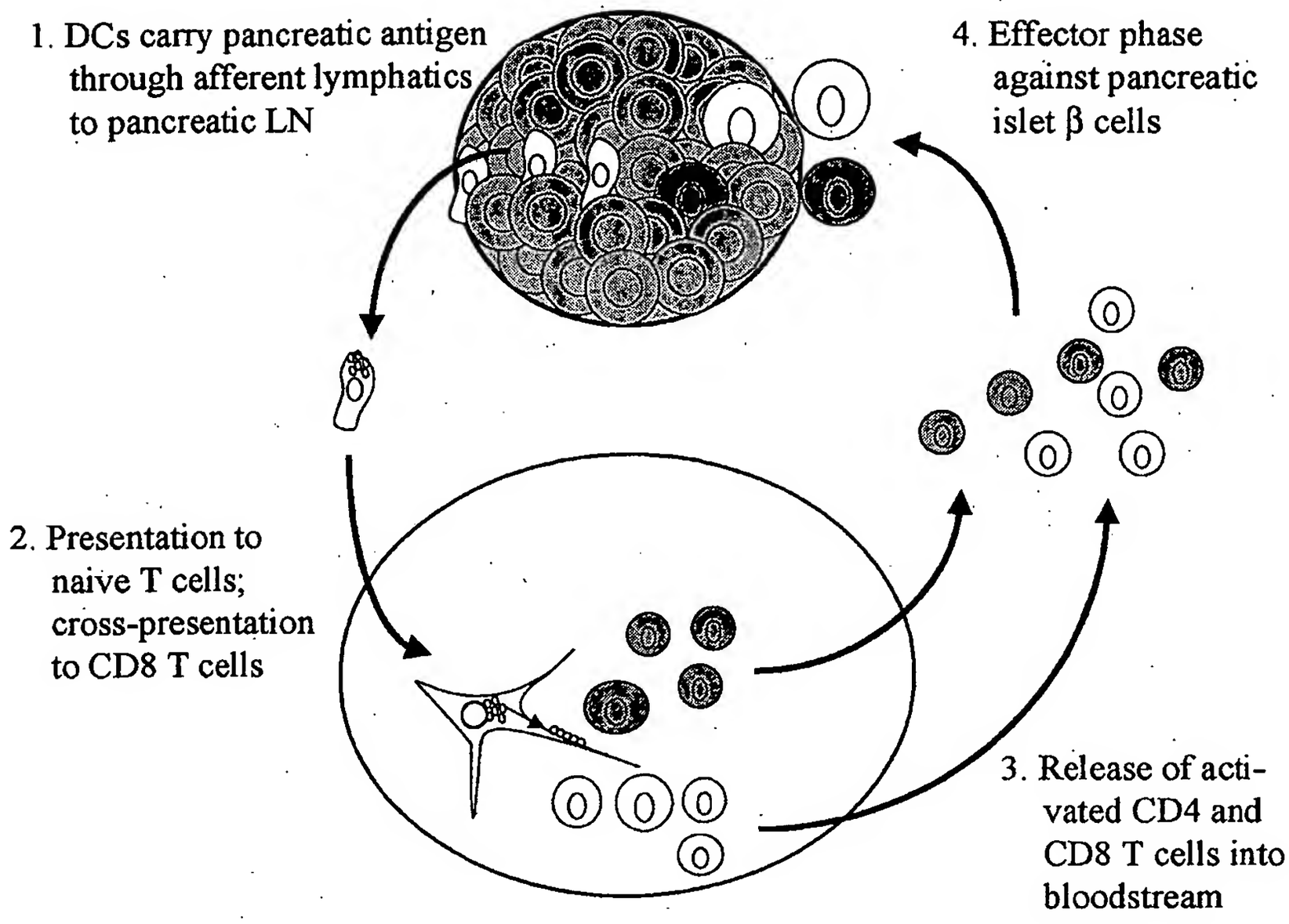

Figure 1. Antigen capture by dendritic cells in islets and their migration to the pancreatic lymph node for presentation of β-cell antigens to T lymphocytes. Antigen-presentation *via* both MHC II and MHC I pathways enables both CD4 and CD8 T cells to be activated. Activated T cells can enter non-lymphoid tissues such as pancreatic islets in search for antigen-expressing target cells (see text for details).

to T cells – both to CD4 and/or CD8 T cells – offers advantages to the immune system in the battle against infections antigens. Only indirect antigen presentation (in the case of CD8 T cells, cross-presentation) allows an immune response to be mounted against viruses that functionally compromise, or simply avoid antigen-presenting cells (103).

These basic mechanisms of antigen presentation, which were uncovered in model systems such as the RIP-mOVA system, were found to be important also for the pathogenesis of diabetes in NOD mice, in which CD8 T cells have emerged as important mediators of disease (113–117). TCR-transgenic NOD mice have been generated, and in many of these, the CD8 T cells destroyed pancreatic islets, whereas the CD4 T cells played more or less relevant helper roles (115, 118, 119). In NOD mice, autoreactive CD4 T cells appear to play an important role in diabetogenesis, and also their activation occurs in the pancreatic lymph nodes (120). Interestingly, presentation of autoantigen to autoreactive CD4 and CD8 T cells is age-dependent. It did not occur in very young animals (120), suggesting that developmental changes in the pancreatic tissue cause the release of autoantigens to the pancreatic lymph node. In this respect, a wave of physiological apoptotic β-cell death, which occurs in rodents at 14–17 days after birth (121), and in humans at birth (122), may be relevant. During such a wave, autoantigens are released from apoptotic islet cells and may activate autoreactive T cells with specificity for β-cell antigens (101, 123). Antigens released from apoptotic cells are taken up particularly well by dendritic cells (124–126) and normally, induce T cell tolerance (127). Why antigen-presentation results in autoimmunity in some individuals is not known, but inflammatory signals caused by accompanying infections (128), differences in the T cell repertoire, genetic susceptibility differences and dysregulated cellular death may be involved. Also, unphysiological β-cell death might cause the release of islet antigens in an immunogenic setting, and induce autoimmunity rather than tolerance (101). Tissue remodelling and replacement of β-cells occur also later. In the RIP-mOVA system, pancreatic tissue antigens were observed to be constantly shuttled to the draining lymph nodes of adult animals, where they were presented to autoreactive T cells (108, 120). These T cells indeed caused diabetes, but only when their precursor frequency was unphysiologically high (103). Physiological numbers of T cells were tolerized

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by deletion before they could destroy all β-cells. Thus, cross-presentation of pancreatic self-antigen led to deletional CD8 T-cell tolerance, which was termed cross-tolerance (129). Cross-tolerance has also been shown in other transgenic systems, and its mechanisms have been elucidated (109). However, in some systems, most notably in those examining antigens derived from the lymphochoriomeningitis virus (LCMV), specific CD8 T cells ignored this self antigen (130) but were not deleted. One possible explanation is that various antigens are handled differently by the immune system. In this case, however, pathogens might easily evolve strategies to escape immune surveillance. An alternative explanation came from studies investigating the influence of antigen dose on cross-presentation. Only high antigen levels were cross-presented and induced cross-tolerance (131–133) while low dose self antigens were ignored, in which case the immune system must rely on ignorance to avoid autoimmunity. Thus, the level of antigen expression appears to determine the mechanism by which CD8 T-cell mediated autoimmunity is avoided.

Ignorance of self antigen allows autoreactive T cells to survive within the T cell repertoire. Such T cells could theoretically unleash their destructive potential if they were activated by other means, for example by pathogens that are similar in antigenic structure. This sword of Damocles has first been demonstrated in the LCMV system, where mice expressing determinants of the LCMV virus in pancreatic islets became diabetic after infection with the virus (134–135). Such a mechanism may explain why immune diseases are often observed after viral infections. Direct evidence for antigenic mimicry as a mechanism of diabetes induction, however, is scarce (136). In contrast to ignorance or to the induction of anergy or TCR downregulation in autoreactive T cells (137, 138) deletion of autoreactive T cells removes the threat of autoimmunity permanently by eliminating potentially harmful effectors. However, this mechanism can also fail, for example if the precursor frequency of the autoreactive CD8 T cells is too high, or when autoreactive CD4 T cells are present (103). Transgenic CD4 T cells specific for islet self-antigens appear to be less efficient as direct mediators of diabetes than CD8 T cells (139, 140). However, they act by delaying the deletion of CD8 effector T cells (139), by mediating their entrance into pancreatic islets (115) or by supporting their effector phase in the islets (107). But also the CD4 T cells themselves are subject to peripheral tolerance. CD4 T cells can be deleted (141) or functionally silenced (137). Also Th1 → Th2 diversion may happen (142) and regulatory $CD25^+$ CD4 T cells, that actively suppress immune responses (143) can be generated.

Molecular mechanisms regulating immune response to islet-antigens

As described above, antigens derived from islet-β-cells are under continuous surveillance by the immune system. Whether this antigen-presentation leads to expansion of islet-specific T lymphocytes and to the development of anti-islet immunity (Fig 1) – or to their silencing – is of crucial importance. Molecular mechanisms underlying this distinction are gradually becoming unravelled. Signalling via cytokine- and costimulatory receptors is of particular importance (144–147). For example, expression of the costimulatory ligand B7.1 or TNF-α on islets, and signalling between CD40L and CD40 can lead to the breakdown of the tolerant state to islet-antigens, as has been shown in NOD mice by antibody treatments and by expression of TNF-α or B7.1 on β-cells (148–150). The action of TNF-α depends, however, on the timing of its action and can also suppress β-cell destruction (149, 150).

The TNF- and TNF-receptor families include several members that may still turn out to be important in the regulation of islet-immunity. A good example is the demonstration that signalling through the TRANCE-RANK receptor-ligand pair is involved in the generation of CD25 + CD4+ regulatory T cells in the pancreatic lymph node (151). These cells were shown to be able to restrain diabetogenic CD8 effector T cells. The existence of regulatory T cells seems to be controlled by costimulatory molecules, because in the NOD mouse, lack of B7-2 or CD28 leads to acceleration of diabetes *via* impaired action of $CD25^+$ CD4 regulatory T cells (152).

Functional silencing, that is 'anergy', is traditionally considered to result from TCR-ligation without simultaneous costimulation but may in fact require costimulation. Accordingly, CTLA-4 is an important negative regulator of the activity of T cells (153), and abrogation of its function leads to acceleration of diabetes in BDC2.5 NOD mice (153). Also regulatory cells including $CD25^+$ CD4 cells (143) and Treg1 cells (154), are partly anergic themselves. Cytokines IL-10 and transforming growth factor (TGF) -β, produced by regulatory T cells, are important in peripheral tolerance via their inhibitory actions on dendritic cell activity and on T cell proliferation and differentiation (155, 156). Which role these regulatory cells play in human disease is not yet clear but is under intensive research.

Receptors that recognize pathogen-associated molecular patterns (PAMP) and are thus called pattern recognition receptors (PRR) are also important in the generation of immune responses (157). These receptors are expressed on cells of the innate immune system including dendritic cells and couple with intracellular signal transduction pathways that reg-

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ulate gene expression. Binding of a ligand (i.e., a foreign pathogen) to such receptors can thus upregulate various functions of dendritic cells. Although it is quite unclear if PRR have any role in the pathophysiology of islet-specific autoimmunity, these receptors and their signalling pathways deserve attention when attempting to manipulate antigen presentation in pancreatic lymph node.

Consequently, costimulatory and/or death-receptors and cytokines expressed by the cells of our immune system form an intricate network of interacting receptors and ligands that influence the fate of islet-reactive T lymphocytes. This network contains a multitude of potential drug targets for attempts to restore tolerance to peripheral tissue antigens such as islet-antigens, some of which are depicted in Figure 2.

Selective modulation of immune response to islet-antigens

Restoration of tolerance to islet β-cells without compromizing general immune function requires that immunomodulation selectively targets the immune response to β-cells. So far, tolerance has been induced experimentally by introducing the antigen in a controlled fashion, i.e., without adjuvants, as a soluble protein and preferably *via* mucosal surfaces or the intravenous route (158, 159). As discussed above, the prospects of these manipulations as immune therapy to diabetes, however, remain very uncertain. Tolerance can also be induced in T cells specific for any given antigen by complexing antigenic peptides with MHC molecules to produce 'custom-made' T cell receptor ligands that are administered without adjuvants and thus in the absence of costimulatory signals (160, 161). This, however, requires detailed knowledge of the immunodominant epitopes of the antigen, and when effective, is likely to be strictly restricted to a narrow specificity of T cells that may not alone be responsible for disease pathogenesis. Thus, multiple T cell receptor ligands would need to be administered unless some of them would be able to induce active regulatory immunity (161).

Current knowledge of the cellular and molecular mechanisms underlying regulation of peripheral tolerance to islet-antigens suggests that new strategies of therapeutic manipulation of the detrimental islet-specific immune response await to be discovered. This would, however, require novel ways of introducing islet-antigens to the immune system in a way that would allow a tolerance-regulating mechanism to be

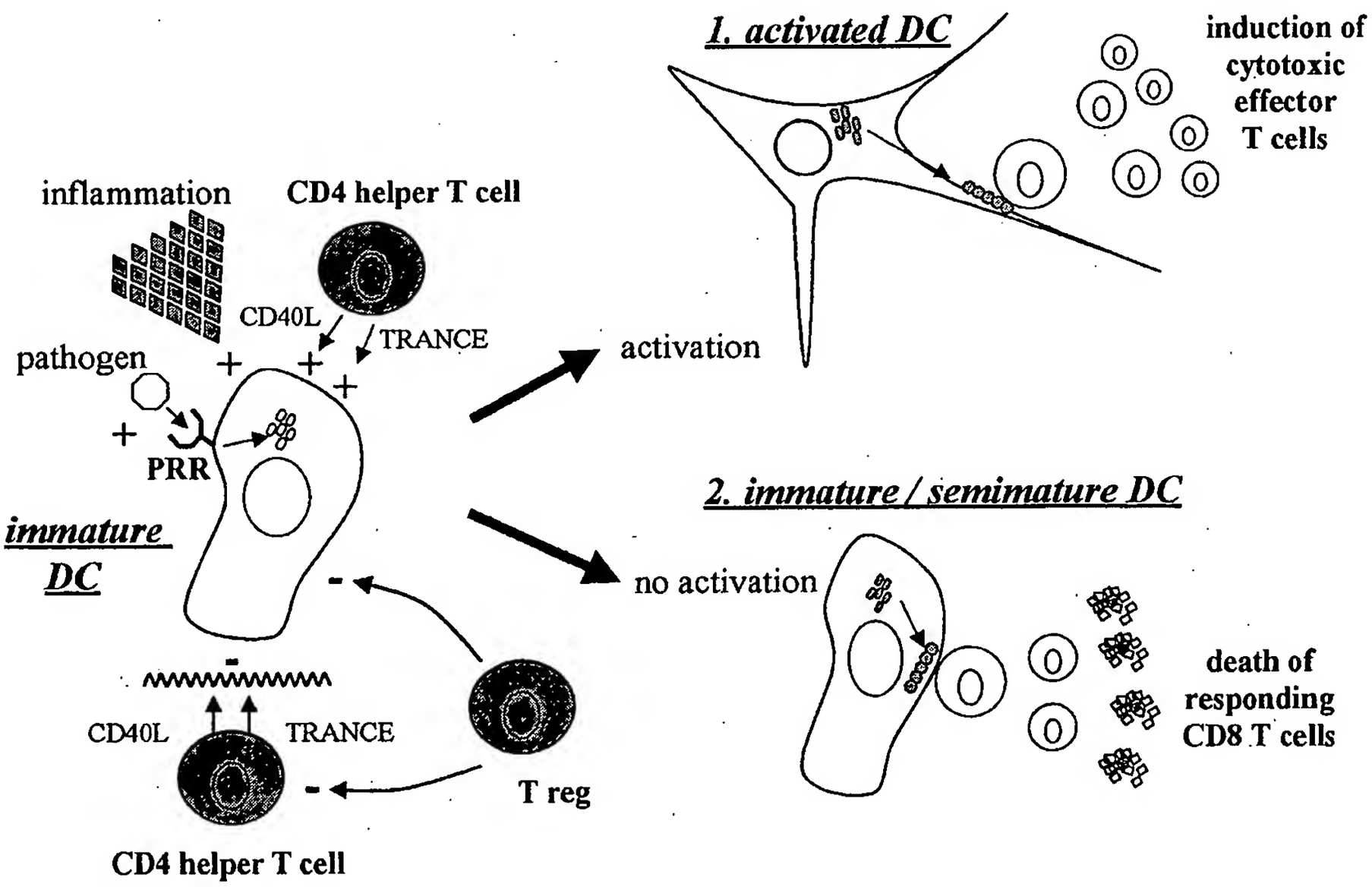

Figure 2. Factors that promote the activation of immature dendritic cell (DC) to become a potent antigen-presenting cell (1. activated DC) after endocytosis of antigen; and the role of regulatory T cells (Treg, e.g., CD25 + CD4 T cells) as opponents of the activation of immature DC (2. immature/semimature DC). PRR = pattern recognition receptor.

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introduced simultaneously with the antigen in a strictly localized manner. Because T cells recognize antigen only when appropriately presented to them, this is possible only during T-cell interaction with an antigen-presenting cell or a target cell. In type 1 diabetes, the target cells (i.e., islet-β cells) are obviously beyond selective manipulation unless xeno-transplantation of pig islets derived from transgenic pigs (whose islets would be made to express immuno-modulatory agents) is considered relevant as a treatment option. Interaction with an APC which is presenting an autoantigen is the other antigen-specific interaction that a diabetogenic T cell commits itself to. Therefore, immunomodulation should probably be targeted here. If this APC cell were made to express an immunomodulatory agent, it could perhaps selectively target this agent to the T cell that is engaged with it *via* its antigen-specific receptor (162). This might re-direct the T cell to a less aggressive behaviour instead of its expansion and maturation into a clone of islet-destructive T cells. The immunomodulatory agent could be a death-inducing ligand such as FasL, TRAIL, TNF or PD-L1 (163–165). Experimental evidence suggests that expression of FasL on dendritic cells renders them tolerogenic (166, 167) although this may not always happen (168). In fact, we have observed that ligation of Fas during T cell contact with dendritic cells costimulates a fraction of the responding T cells (169) which could complicate its effects as a death-ligand. For other death-receptor ligands, data do not yet exist.

Alternatively, the immunomodulatory agent could be a cytokine such as IL-10 or TGF-β, because these cytokines regulate the costimulatory activity of the antigen-presenting cell and proliferation and polarity of the responding T cell as discussed above (155, 156). For therapeutic purposes, this type of targeted immunomodulation could be achieved by *in vitro*-treatment of autologous antigen-presenting cells with the immunomodulatory agent together with the antigen before re-injection, or by genetic modification to make these cells express the immunomodulatory agent themselves. Also, although B lymphocytes as well as monocytes can present antigen to T cells, dendritic cells would likely suit best to be used as modified antigen-presenting cells. This is because only dendritic cells are able to induce a response both in naïve and antigen-experienced T cells (170), and because B lymphocytes have an intrinsic property of directing islet-reactive T cells into diabetogenic behaviour at least in the NOD mouse (171, 172).

Modification of antigen-presentation from dendritic cells – a therapeutic option?

Dendritic cells can be propagated from peripheral blood of humans with the aid of cytokines granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 (173, 174). Such dendritic cells could be treated *in vitro* with an immunomodulatory agent or perhaps transfected with a gene construct to make them express the immunomodulatory agent themselves and pulsed with antigen before re-injection back into the same individual. *In vitro* cultured and antigen-pulsed dendritic cells from healthy individuals can, in fact, either induce a potent immune response or tolerance depending on their prior *in vitro*-treatment when re-injected (175, 176). The limitations of this approach would come from the amount of work and potential safety risks introduced by *in vitro* propagation of autologous cells. These limitations would not apply to the use of purified DNA as a 'vaccine' (177). The discovery that injecting 'naked' DNA can induce immunity was made a decade ago (178), and vaccination with 'naked' DNA represents a promising strategy for inducing cell-mediated immune responses (including cytotoxic CD8 T cells) (177, 178). Purified plasmid DNA encoding both an autoantigen and an immunomodulatory agent could therefore work as a 'vaccine' that might elicit a modified immune response resulting in tolerance instead of effective cell-mediated immunity. Unlike cells that need to be autologous, a vaccine consisting of recombinant DNA once validated could potentially be applied to individuals of a diverse genetic background (i.e., irrespective of MHC haplotypes or other disparate features). Thus, a 'vaccine' for people at risk of developing T1DM could perhaps become available on a large scale. The safety risks of introducing foreign recombinant DNA would probably be small if vectors that are able to incorporate into host genome (i.e., retroviral vectors) would not be applied.

In the NOD mouse, a few studies already exist in which DNA-plasmids encoding insulin, its B chain, GAD or hsp 60 either alone or together with a plasmid encoding a regulatory cytokine (e.g., IL-4) have been injected as a 'vaccine' to induce tolerance (47, 179–181). DNA injected as a 'vaccine' is taken up by cells in the tissue including dendritic cells which express the antigen and present it to T cells (182). In many cases this has resulted in regulatory immunity and protection from disease. This is noteworthy, given the capacity of DNA-vaccination to induce effective cytotoxic T-cell immunity in tumour and viral disease models (183, 184). Hence, the control of possible adverse immune reactions deserves careful attention when using this strategy. We believe that simultaneous expression of the autoantigen with an immunomodulatory agent could be a possible way to better control such adverse reactions and to target immunomodulation most effectively to pathogenic T cells. Intensive research is

yet needed in models such as the RIPmOVA -model and the NOD mouse to identify the most effective immunomodulatory molecules and the most relevant islet-autoantigens that these should be combined with.

A promising strategy to induce tolerance towards a defined antigen is to make a fusion protein consisting of the antigen and an antibody to an endocytosis receptor expressed on immature dendritic cells. Accordingly, the model antigen ovalbumin when conjugated to an antibody against the DEC-205 receptor, induced antigen presentation in dendritic cells that remained in an immature state (185). Presentation of antigen *via* immature dendritic cells rendered mice tolerant to subsequent challenge with the same antigen showing that targeting the antigen thoughtfully to the immune system may elicit antigen-specific tolerance.

We have discussed here the role of animal models in development of therapeutic strategies to induce immune tolerance to islet-autoantigens and envisioned some novel approaches to modify antigen-presentation from dendritic cells to induce tolerance (Fig 3). These strategies and approaches are currently under investigation in many laboratories including our own laboratories. Via innovative and careful work in animal models of islet-autoimmunity some of these approaches may be transformed into strategies that could be applied as specific immunotherapy to type 1 diabetes. Compared to treatment with general immunomodulatory or immunosuppressive agents, or other general, although certainly effective, treatment options like bone marrow reconstitution (186), tolerance-promoting presentation of autoantigens to the immune system would offer a worthwhile choice.

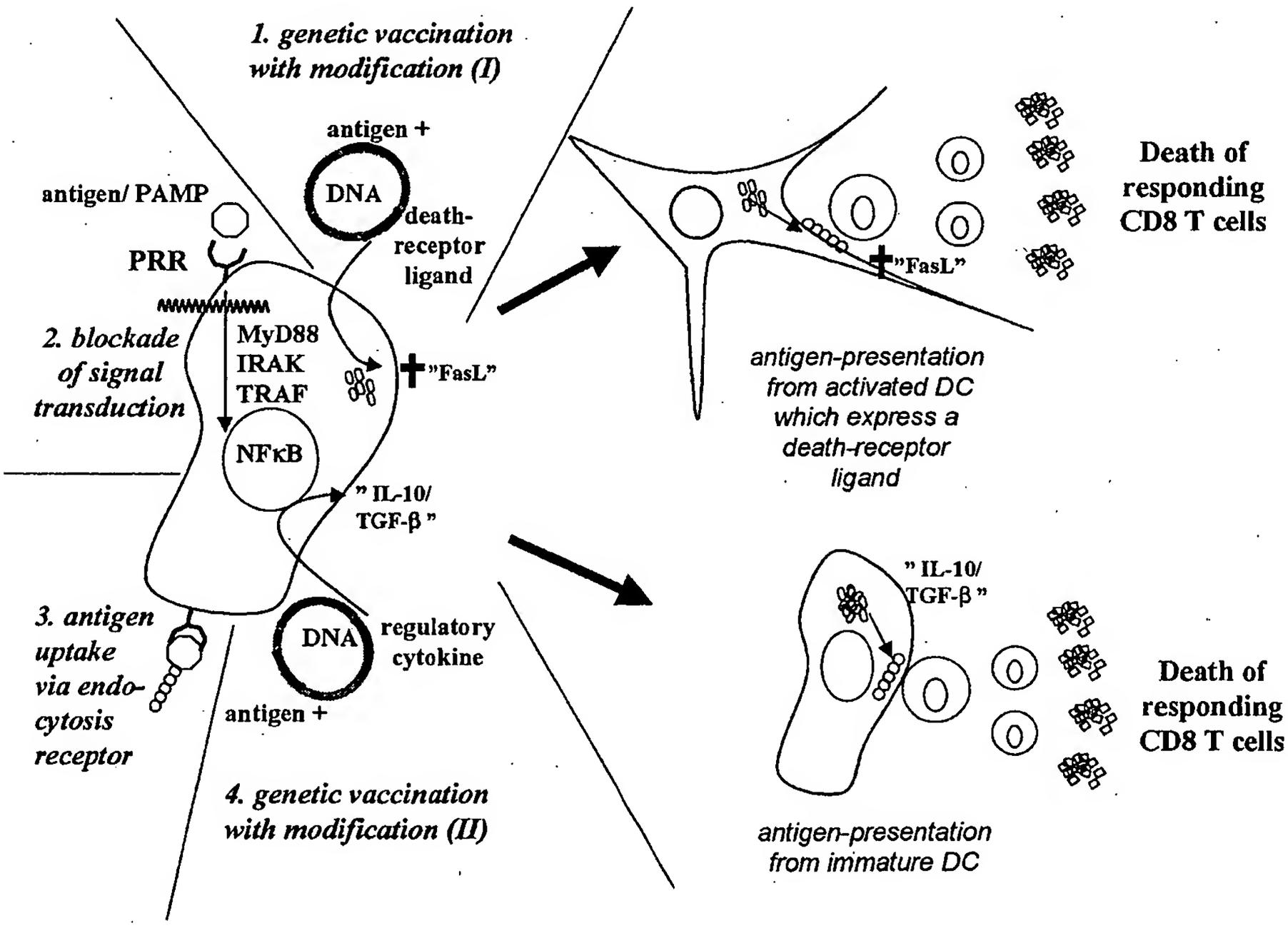

Figure 3. Four potential strategies for manipulating antigen-presentation in dendritic cells to induce tolerance. 1. Genetic (i.e. DNA) vaccination with a plasmid that encodes antigen and a death-receptor ligand (FasL, TRAIL, PD-L1/2); 2. Blockade of signal transduction from pathogen recognition receptor (PRR) by e.g., genetic modification of DC or antisense oligonucleotides; 3. Conjugation of antigen to a structure that binds to an endocytosis receptor (e.g., DEC-205) expressed on immature DC; 4. Genetic (i.e., DNA) vaccination with a plasmid that induces production of a regulatory cytokine (TGF-β, IL-10).

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References

1. Knip M. Natural course of preclinical type 1 diabetes. Horm Res 2002;57 Suppl 1:6–11.
2. Notkins AL. Immunologic and genetic factors in type 1 diabetes. J Biol Chem 2002;277:43545–8.
3. Roep BO. T-cell reactivity to beta-cell antigens in human insulin-dependent (type 1) diabetes mellitus. Implications for diagnosis and therapy. Clin Rev Allergy Immunol 2000;19: 265–75.
4. Bottazzo GF, Dean BM, McNally JM, MacKay EH, Swift PG, Gamble DR. In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. N Engl J Med 1985;313:353–60.
5. Hanninen A, Jalkanen S, Salmi M, Toikkanen S, Nikolakaros G, Simell O. Macrophages, T cell receptor usage, and endothelial cell activation in the pancreas at the onset of insulin-dependent diabetes mellitus. J Clin Invest 1992;90: 1901–10.
6. Itoh N, Hanafusa T, Miyazaki A, Miyagawa J, Yamagata K, Yamamoto K, et al. Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. J Clin Invest 1993;92:2313–22.
7. Sutherland DE, Goetz FC, Sibley RK. Recurrence of disease in pancreas transplants. Diabetes 1989;38 Suppl 1:85–7.
8. Assan R, Bach JF, Czernichow P, Dupre J, Stiller CR, Eisenbarth GS, et al. Immunosuppressive drugs in diabetes. Lancet 1986;2:1097.
9. Gottlieb PA, Hayward AR. Cytokine and immunosuppressive therapies of type 1 diabetes mellitus. Endocrinol Metab Clin North Am 2002;31:477–95.
10. Akerblom HK, Knip M. Putative environmental factors in Type 1 diabetes. Diabetes Metab Rev 1998;14:31–67.
11. Knip M, Akerblom HK. Environmental factors in the pathogenesis of type 1 diabetes mellitus. Exp Clin Endocrinol Diabetes 1999;107 Suppl 3:S93–100.
12. Atkinson MA, Eisenbarth GS. Type 1 diabetes:new perspectives on disease pathogenesis and treatment. Lancet 2001;358: 221–9.
13. Atkinson MA, Maclaren NK. The pathogenesis of insulin-dependent diabetes mellitus. N Engl J Med 1994;331:1428–36.
14. Atkinson MA, Maclaren NK. Islet cell autoantigens in insulin-dependent diabetes. J Clin Invest 1993;92:1608–16.
15. Harrison LC. Islet cell antigens in insulin-dependent diabetes: Pandora's box revisited. Immunol Today 1992;13:348–52.
16. Leslie RD, Atkinson MA, Notkins AL. Autoantigens IA-2 and GAD in Type I (insulin-dependent) diabetes. Diabetologia 1999;42:3–14.
17. Atkinson MA, Leiter EH. The NOD mouse model of type 1 diabetes: as good as it gets? Nat Med 1999;5:601–4.
18. Mathis DJ, Benoist C, Williams VE, 2nd, Kanter M, McDevitt HO. Several mechanisms can account for defective E alpha gene expression in different mouse haplotypes. Proc Natl Acad Sci USA 1983;80:273–7.
19. Lynch DM, Kay PH. Studies on the polymorphism of the fifth component of complement in laboratory mice. Exp Clin Immunogenet 1995;12:253–60.
20. Kikutani H, Makino S. The murine autoimmune diabetes model: NOD and related strains. Adv Immunol 1992;51:285–322.
21. Yoshida K, Kikutani H. Genetic and immunological basis of autoimmune diabetes in the NOD mouse. Rev Immunogenet 2000;2:140–6.
22. Adorini L, Gregori S, Harrison LC. Understanding autoimmune diabetes: insights from mouse models. Trends Mol Med 2002;8:31–8.
23. Hayward AR, Shreiber M. Neonatal injection of CD3 antibody into nonobese diabetic mice reduces the incidence of insulitis and diabetes. J Immunol 1989;143:1555–9.
24. Chatenoud L, Primo J, Bach JF. CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. J Immunol 1997;158:2947–54.
25. Varey AM, Hutchings P, O'Reilly L, Hussell T, Waldmann H, Simpson E, et al. The development of insulin-dependent diabetes mellitus in non-obese diabetic mice: the role of CD4+ and CD8+ T cells. Biochem Soc Trans 1991;19:187–91.
26. Sempe P, Bedossa P, Richard MF, Villa MC, Bach JF, Boitard C. Anti-alpha/beta T cell receptor monoclonal antibody provides an efficient therapy for autoimmune diabetes in nonobese diabetic (NOD) mice. Eur J Immunol 1991;21: 1163–9.
27. Lenschow DJ, Ho SC, Sattar H, Rhee L, Gray G, Nabavi N, et al. Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. J Exp Med 1995;181:1145–55.
28. Abu-Hadid MM, Lazarovits AI, Madrenas J. Prevention of diabetes mellitus in the non-obese diabetic mouse strain with monoclonal antibodies against the CD45RB molecule. Autoimmunity 2000;32:73–6.
29. Balasa B, Krahl T, Patstone G, Lee J, Tisch R, McDevitt HO, et al. CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in nonobese diabetic mice. J Immunol 1997;159:4620–7.
30. Baron JL, Reich EP, Visintin I, Janeway CA, Jr. The pathogenesis of adoptive murine autoimmune diabetes requires an interaction between alpha 4-integrins and vascular cell adhesion molecule-1. J Clin Invest 1994;93:1700–8.
31. Yang XD, Michie SA, Tisch R, Karin N, Steinman L, McDevitt HO. A predominant role of integrin alpha 4 in the spontaneous development of autoimmune diabetes in non-obese diabetic mice. Proc Natl Acad Sci USA 1994;91: 12604–8.
32. Hanninen A, Jaakkola I, Jalkanen S. Mucosal addressin is required for the development of diabetes in nonobese diabetic mice. J Immunol 1998;160:6018–25.
33. Moriyama H, Yokono K, Amano K, Nagata M, Hasegawa Y, Okamoto N, et al. Induction of tolerance in murine autoimmune diabetes by transient blockade of leukocyte function-associated antigen-1/intercellular adhesion molecule-1 pathway. J Immunol 1996;157:3737–43.
34. Calcinaro F, Gambelunghe G, Lafferty KJ. Protection from autoimmune diabetes by adjuvant therapy in the non-obese diabetic mouse: the role of interleukin-4 and interleukin-10. Immunol Cell Biol 1997;75:467–71.
35. Overbergh L, Decallonne B, Waer M, Rutgeerts O, Valckx D, Casteels KM, et al. 1alpha,25-dihydroxyvitamin D3 induces an autoantigen-specific T-helper 1/T-helper 2 immune shift in NOD mice immunized with GAD65 (p524-543). Diabetes 2000;49:1301–7.
36. Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, et al. Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. Nat Med 2001;7:1057–62.
37. Tominaga Y, Nagata M, Yasuda H, Okamoto N, Arisawa K, Moriyama H, et al. Administration of IL-4 prevents autoimmune diabetes but enhances pancreatic insulitis in NOD mice. Clin Immunol Immunopathol 1998;86:209–18.
38. Pennline KJ, Roque-Gaffney E, Monahan M. Recombinant

 ISSN 0785-3890

human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. Clin Immunol Immunopathol 1994;71:169–75.
39. Zheng XX, Steele AW, Hancock WW, Stevens AC, Nickerson PW, Roy-Chaudhury P, et al. A noncytolytic IL-10/Fc fusion protein prevents diabetes, blocks autoimmunity, and promotes suppressor phenomena in NOD mice. J Immunol 1997;158:4507–13.
40. Reddy S, Bibby NJ, Elliott RB. Early nicotinamide treatment in the NOD mouse: effects on diabetes and insulitis suppression and autoantibody levels. Diabetes Res 1990;15: 95–102.
41. Atkinson MA, Maclaren NK, Luchetta R. Insulitis and diabetes in NOD mice reduced by prophylactic insulin therapy. Diabetes 1990;39:933–7.
42. Muir A, Peck A, Clare-Salzler M, Song Y-H, Cornelius J, Luchetta R, et al. Insulin immunization of nonobese diabetic mice induces a protective insulitis characterized by diminished intraislet interferon-γ transcription. J Clin Invest 1995; 95:628–34.
43. Zhang ZJ, Davidson L, Eisenbarth G, Weiner HL. Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. Proc Natl Acad Sci USA 1991;88: 10252–6.
44. Bergerot I, Fabien N, Mayer A, Thivolet C. Active suppression of diabetes after oral administration of insulin is determined by antigen dosage. Ann N Y Acad Sci 1996;778: 362–7.
45. Harrison LC, Dempsey-Collier M, Kramer DR, Takahashi K. Aerosol insulin induces regulatory CD8 gamma delta T cells that prevent murine insulin-dependent diabetes. J Exp Med 1996;184:2167–74.
46. Daniel D, Wegmann DR. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9–23). Proc Natl Acad Sci USA 1996;93:956–60.
47. Urbanek-Ruiz I, Ruiz PJ, Paragas V, Garren H, Steinman L, Fathman CG. Immunization with DNA encoding an immunodominant peptide of insulin prevents diabetes in NOD mice. Clin Immunol 2001;100:164–71.
48. Tian J, Atkinson MA, Clare-Salzler M, Herschenfeld A, Forsthuber T, Lehmann PV. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. J Exp Med 1996;183:1561–7.
49. Balasa B, Boehm BO, Fortnagel A, Karges W, Van Gunst K, Jung N, et al. Vaccination with glutamic acid decarboxylase plasmid DNA protects mice from spontaneous autoimmune diabetes and B7/CD28 costimulation circumvents that protection. Clin Immunol 2001;99:241–52.
50. Tisch R, Wang B, Weaver DJ, Liu B, Bui T, Arthos J, et al. Antigen-specific mediated suppression of beta cell autoimmunity by plasmid DNA vaccination. J Immunol 2001; 166:2122–32.
51. Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, McDevitt HO. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Nature 1993;366:72–5.
52. Elias D, Cohen IR. Peptide therapy for diabetes in NOD mice. Lancet 1994;343:704–6.
53. Blanas E, Carbone FR, Allison J, Miller JF, Heath WR. Induction of autoimmune diabetes by oral administration of autoantigen. Science 1996;274:1707–9.
54. Hanninen A, Braakhuis A, Heath WR, Harrison LC. Mucosal antigen primes diabetogenic cytotoxic T-lymphocytes regardless of dose or delivery route. Diabetes 2001;50: 771–5.
55. Hanninen A, Martinez NR, Davey GM, Heath WR, Harrison LC. Transient blockade of CD40 ligand dissociates pathogenic from protective mucosal immunity. J Clin Invest 2002;109:261–7.
56. von Herrath MG, Whitton JL. DNA vaccination to treat autoimmune diabetes. Ann Med 2000;32:285–92.
57. Woodle ES, Bluestone JA, Zivin RA, Jolliffe LK, Auger J, Xu D, et al. Humanized, nonmitogenic OKT3 antibody, huOKT3 gamma(Ala-Ala): initial clinical experience. Transplant Proc 1998;30:1369–70.
58. Herold KC, Bluestone JA, Montag AG, Parihar A, Wiegner A, Gress RE, et al. Prevention of autoimmune diabetes with nonactivating anti-CD3 monoclonal antibody. Diabetes 1992;41:385–91.
59. Chatenoud L, Thervet E, Primo J, Bach JF. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci USA 1994;91: 123–7.
60. Smith JA, Tso JY, Clark MR, Cole MS, Bluestone JA. Nonmitogenic anti-CD3 monoclonal antibodies deliver a partial T cell receptor signal and induce clonal anergy. J Exp Med 1997;185:1413–22.
61. Smith JA, Tang Q, Bluestone JA. Partial TCR signals delivered by FcR-nonbinding anti-CD3 monoclonal antibodies differentially regulate individual Th subsets. J Immunol 1998;160:4841–9.
62. Elias D, Meilin A, Ablamunits V, Birk OS, Carmi P, Konen-Waisman S, et al. Hsp60 peptide therapy of NOD mouse diabetes induces a Th2 cytokine burst and downregulates autoimmunity to various beta-cell antigens. Diabetes 1997; 46:758–64.
63. Raz I, Elias D, Avron A, Tamir M, Metzger M, Cohen IR. Beta-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial. Lancet 2001;358: 1749–53.
64. Gotfredsen CF, Buschard K, Frandsen EK. Reduction of diabetes incidence of BB Wistar rats by early prophylactic insulin treatment of diabetes-prone animals. Diabetologia 1985;28:933–5.
65. Keller RJ, Eisenbarth GS, Jackson RA. Insulin prophylaxis in individuals at high risk of type I diabetes. Lancet 1993;341: 927–8.
66. Miller A, Lider O, Weiner HL. Antigen-driven bystander suppression after oral administration of antigens. J Exp Med 1991;174:791–8.
67. Gallichan WS, Rosenthal KL. Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization. J Exp Med 1996;184:1879–90.
68. Heller B, Wang ZQ, Wagner EF, Radons J, Burkle A, Fehsel K, et al. Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. J Biol Chem 1995;270:11176–80.
69. Pozzilli P, Visalli N, Signore A, Baroni MG, Buzzetti R, Cavallo MG, et al. Double blind trial of nicotinamide in recent-onset IDDM (the IMDIAB III study). Diabetologia 1995;38:848–52.
70. Chase HP, Butler-Simon N, Garg S, McDuffie M, Hoops SL, O'Brien D. A trial of nicotinamide in newly diagnosed patients with type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1990;33:444–6.
71. Mendola G, Casamitjana R, Gomis R. Effect of nicotinamide therapy upon B-cell function in newly diagnosed type 1 (insulin-dependent) diabetic patients. Diabetologia 1989;32: 160–2.
72. Lampeter EF, Klinghammer A, Scherbaum WA, Heinze E, Haastert B, Giani G, et al. The Deutsche Nicotinamide Intervention Study: an attempt to prevent type 1 diabetes. DENIS Group. Diabetes 1998;47:980–4.

 ISSN 0785-3890

73. Elliott RB, Pilcher CC, Fergusson DM, Stewart AW. A population based strategy to prevent insulin-dependent diabetes using nicotinamide. J Pediatr Endocrinol Metab 1996;9:501–9.
74. Brod SA, Malone M, Darcan S, Papolla M, Nelson L. Ingested interferon alpha suppresses type I diabetes in non-obese diabetic mice. Diabetologia 1998;41:1227–32.
75. Brod SA, Atkinson M, Lavis VR, Brosnan PG, Hardin DS, Orlander PR, et al. Ingested IFN-alpha preserves residual beta cell function in type 1 diabetes. J Interferon Cytokine Res 2001;21:1021–30.
76. Fleischmann WR, Jr, Koren S, Fleischmann CM. Orally administered interferons exert their white blood cell suppressive effects via a novel mechanism. Proc Soc Exp Biol Med 1992;201:200–7.
77. Stewart TA, Hultgren B, Huang X, Pitts-Meek S, Hully J, MacLachlan NJ. Induction of type I diabetes by interferon-alpha in transgenic mice. Science 1993;260:1942–6.
78. Cunningham-Rundles S, Ahrne S, Bengmark S, Johann-Liang R, Marshall F, Metakis L, et al. Probiotics and immune response. Am J Gastroenterol 2000;95:S22–5.
79. Kukreja A, Cost G, Marker J, Zhang C, Sun Z, Lin-Su K, et al. Multiple immuno-regulatory defects in type-1 diabetes. J Clin Invest 2002;109:131–40.
80. Wilson SB, Kent SC, Patton KT, Orban T, Jackson RA, Exley M, et al. Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. Nature 1998; 391:177–81.
81. Lee PT, Putnam A, Benlagha K, Teyton L, Gottlieb PA, Bendelac A. Testing the NKT cell hypothesis of human IDDM pathogenesis. J Clin Invest 2002;110:793–800.
82. Hammond KJ, Poulton LD, Palmisano LJ, Silveira PA, Godfrey DI, Baxter AG. alpha/beta-T cell receptor (TCR)+CD4-CD8- (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. J Exp Med 1998;187:1047–56.
83. Lehuen A, Lantz O, Beaudoin L, Laloux V, Carnaud C, Bendelac A, et al. Overexpression of natural killer T cells protects Valpha14- Jalpha281 transgenic nonobese diabetic mice against diabetes. J Exp Med 1998;188:1831–9.
84. Matsuzaki T, Nagata Y, Kado S, Uchida K, Kato I, Hashimoto S, et al. Prevention of onset in an insulin-dependent diabetes mellitus model, NOD mice, by oral feeding of Lactobacillus casei. APMIS 1997;105:643–9.
85. Hessle C, Hanson LA, Wold AE. Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Clin Exp Immunol 1999;116:276–82.
86. Haskins K, Wegmann D. Diabetogenic T-cell clones. Diabetes 1996;45:1299–305.
87. Wegmann DR, Norbury-Glaser M, Daniel D. Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice. Eur J Immunol 1994;24:1853–7.
88. Daniel D, Gill RG, Schloot N, Wegmann D. Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. Eur J Immunol 1995;25:1056–62.
89. Alleva DG, Gaur A, Jin L, Wegmann D, Gottlieb PA, Pahuja A, et al. Immunological characterization and therapeutic activity of an altered-peptide ligand, NBI-6024, based on the immunodominant type 1 diabetes autoantigen insulin B-chain (9–23) peptide. Diabetes 2002;51:2126–34.
90. Nicholson LB, Kuchroo VK. T cell recognition of self and altered self antigens. Crit Rev Immunol 1997;17:449–62.
91. Young DA, Lowe LD, Booth SS, Whitters MJ, Nicholson L, Kuchroo VK, et al. IL-4, IL-10, IL-13, and TGF-beta from an altered peptide ligand-specific Th2 cell clone down-regulate adoptive transfer of experimental autoimmune encephalomyelitis. J Immunol 2000;164:3563–72.
92. Veldman CM, Cantorna MT, DeLuca HF. Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system. Arch Biochem Biophys 2000;374:334–8.
93. Lemire JM, Archer DC, Beck L, Spiegelberg HL. Immunosuppressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1 functions. J Nutr 1995;125:1704S–8S.
94. Penna G, Adorini L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. J Immunol 2000;164:2405–11.
95. Griffin MD, Lutz W, Phan VA, Bachman LA, McKean DJ, Kumar R. Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo. Proc Natl Acad Sci USA 2001;98:6800–5.
96. Mathieu C, Waer M, Casteels K, Laureys J, Bouillon R. Prevention of type I diabetes in NOD mice by nonhypercalcemic doses of a new structural analog of 1,25-dihydroxyvitamin D3, KH1060. Endocrinology 1995;136:866–72.
97. Adorini L, Penna G, Giarratana N, Uskokovic M. Tolerogenic dendritic cells induced by vitamin D receptor ligands enhance regulatory T cells inhibiting allograft rejection and autoimmune diseases. J Cell Biochem 2003;88:227–33.
98. Karges W, Hammond-McKibben D, Cheung RK, Visconti M, Shibuya N, Kemp D, et al. Immunological aspects of nutritional diabetes prevention in NOD mice: a pilot study for the cow's milk-based IDDM prevention trial. Diabetes 1997;46:557–64.
99. Bodington MJ, McNally PG, Burden AC. Cow's milk and type 1 childhood diabetes: no increase in risk. Diabet Med 1994;11:663–5.
100. Fava D, Leslie RD, Pozzilli P. Relationship between dairy product consumption and incidence of IDDM in childhood in Italy. Diabetes Care 1994;17:1488–90.
101. Mathis D, Vence L, Benoist C. beta-Cell death during progression to diabetes. Nature 2001;414:792–8.
102. Miller JFAP, Flavell RA. T cell tolerance and autoimmunity in transgenic models of central and peripheral tolerance. Curr Opin Immunol 1994;6:892–9.
103. Kurts C. Cross-presentation: inducing CD8 T cell immunity and tolerance. J Mol Med 2000;78:326–32.
104. Mintern J, Li M, Davey GM, Blanas E, Kurts C, Carbone FR, et al. The use of carboxyfluorescein diacetate succinimidyl ester to determine the site, duration and cell type responsible for antigen presentation in vivo. Immunol Cell Biol 1999;77:539–43.
105. Blanas E, Davey GM, Carbone FR, Heath WR. A bone marrow-derived APC in the gut-associated lymphoid tissue captures oral antigens and presents them to both CD4+ and CD8+ T cells. J Immunol 2000;164:2890–6.
106. Arnold B, Goodnow C, Hengartner H, Hammerling G. The coming of transgenic mice: tolerance and immune reactivity. Immunol Today 1990;11:69–72.
107. Heath WR, Allison J, Hoffmann MW, Schonrich G, Hammerling G, Arnold B, et al. Autoimmune diabetes as a consequence of locally produced interleukin-2. Nature 1992; 359:547–9.
108. Kurts C, Heath WR, Carbone FR, Allison J, Miller JF, Kosaka H. Constitutive class I-restricted exogenous presentation of self antigens in vivo. J Exp Med 1996;184:923–30.
109. Kreuwel HT, Aung S, Silao C, Sherman LA. Memory CD8(+) T cells undergo peripheral tolerance. Immunity 2002;17:73–81.
110. Huang AY, Golumbek P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Role of bone marrow-derived cells in

 ISSN 0785-3890

presenting MHC class I-restricted tumor antigens. Science 1994;264:961–5.
111. Sigal LJ, Crotty S, Andino R, Rock KL. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. Nature 1999;398:77–80.
112. Mueller SN, Jones CM, Smith CM, Heath WR, Carbone FR. Rapid cytotoxic T lymphocyte activation occurs in the draining lymph nodes after cutaneous herpes simplex virus infection as a result of early antigen presentation and not the presence of virus. J Exp Med 2002;195:651–6.
113. Liblau RS, Wong FS, Mars LT, Santamaria P. Autoreactive CD8 T cells in organ-specific autoimmunity: emerging targets for therapeutic intervention. Immunity 2002;17:1–6.
114. Wong FS, Karttunen J, Dumont C, Wen L, Visintin I, Pilip IM, et al. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. Nat Med 1999;5:1026–31.
115. Verdaguer J, Schmidt D, Amrani A, Anderson B, Averill N, Santamaria P. Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. J Exp Med 1997;186: 1663–76.
116. DiLorenzo TP, Graser RT, Ono T, Christianson GJ, Chapman HD, Roopenian DC, et al. Major histocompatibility complex class I-restricted T cells are required for all but the end stages of diabetes development in nonobese diabetic mice and use a prevalent T cell receptor alpha chain gene rearrangement. Proc Natl Acad Sci USA 1998;95:12538–43.
117. Hamilton-Williams EE, Serreze DV, Charlton B, Johnson EA, Marron MP, Mullbacher A, et al. Transgenic rescue implicates beta2-microglobulin as a diabetes susceptibility gene in nonobese diabetic (NOD) mice. Proc Natl Acad Sci USA 2001;98:11533–8.
118. Graser RT, DiLorenzo TP, Wang F, Christianson GJ, Chapman HD, Roopenian DC, et al. Identification of a CD8 T cell that can independently mediate autoimmune diabetes development in the complete absence of CD4 T cell helper functions. J Immunol 2000;164:3913–8.
119. Kanagawa O, Shimizu J, Vaupel BA. Thymic and postthymic regulation of diabetogenic CD8 T cell development in TCR transgenic nonobese diabetic (NOD) mice. J Immunol 2000; 164:5466–73.
120. Hoglund P, Mintern J, Waltzinger C, Heath W, Benoist C, Mathis D. Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. J Exp Med 1999;189:331–9.
121. Finegood DT, Scaglia L, Bonner-Weir S. Dynamics of beta-cell mass in the growing rat pancreas. Estimation with a simple mathematical model. Diabetes 1995;44:249–56.
122. Kassem SA, Ariel I, Thornton PS, Scheimberg I, Glaser B. Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. Diabetes 2000;49:1325–33.
123. Trudeau JD, Dutz JP, Arany E, Hill DJ, Fieldus WE, Finegood DT. Neonatal beta-cell apoptosis: a trigger for autoimmune diabetes? Diabetes 2000;49:1–7.
124. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature 1998;392:86–9.
125. Kurts C, Miller JF, Subramaniam RM, Carbone FR, Heath WR. Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. J Exp Med 1998; 188:409–14.
126. Iyoda T, Shimoyama S, Liu K, Omatsu Y, Akiyama Y, Maeda Y, et al. The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. J Exp Med 2002;195:1289–302.
127. Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med 2000;191:411–6.
128. Vezys V, Lefrancois L. Cutting edge: inflammatory signals drive organ-specific autoimmunity to normally cross-tolerizing endogenous antigen. J Immunol 2002;169:6677–80.
129. Heath WR, Carbone FR. Cross-presentation in viral immunity and self-tolerance. Nat Rev Immunol 2001;1:126–34.
130. Ochsenbein AF, Klenerman P, Karrer U, Ludewig B, Pericin M, Hengartner H, et al. Immune surveillance against a solid tumor fails because of immunological ignorance. Proc Natl Acad Sci USA 1999;96:2233–8.
131. Morgan DJ, Kreuwel HT, Sherman LA. Antigen concentration and precursor frequency determine the rate of CD8+ T cell tolerance to peripherally expressed antigens. J Immunol 1999;163:723–7.
132. Kurts C, Sutherland RM, Davey G, Li M, Lew AM, Blanas E, et al. CD8 T cell ignorance or tolerance to islet antigens depends on antigen dose. Proc Natl Acad Sci USA 1999;96: 12703–7.
133. Ohashi PS. T-cell signalling and autoimmunity: molecular mechanisms of disease. Nat Rev Immunol 2002;2:427–38.
134. Ohashi PS, Oehen S, Buerki K, Pircher H, Ohashi CT, Odermatt B, et al. Ablation of 'tolerance' and induction of diabetes by virus infection in viral antigen transgenic mice. Cell 1991;65:305–17.
135. Oldstone MB, Nerenberg M, Southern P, Price J, Lewicki H. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell 1991;65:319–31.
136. Benoist C, Mathis D. Autoimmunity provoked by infection: how good is the case for T cell epitope mimicry? Nat Immunol 2001;2:797–801.
137. Schwartz RH. T Cell Anergy. Annu Rev Immunol 2003;21: 305–34.
138. Schonrich G, Kalinke U, Momburg F, Malissen M, Schmitt-Verhulst AM, Malissen B, et al. Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. Cell 1991;65:293–304.
139. Kurts C, Carbone FR, Barnden M, Blanas E, Allison J, Heath WR, et al. CD4+ T cell help impairs CD8+ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity. J Exp Med 1997;186:2057–62.
140. Adler AJ, Marsh DW, Yochum GS, Guzzo JL, Nigam A, Nelson WG, et al. CD4+ T cell tolerance to parenchymal self-antigens requires presentation by bone marrow-derived antigen-presenting cells. J Exp Med 1998;187:1555–64.
141. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996;383:787–93.
142. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7:145–73.
143. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, et al. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol Rev 2001;182:18–32.
144. Grewal IS, Grewal KD, Wong FS, Wang H, Picarella DE, Janeway CA, Jr., et al. Expression of transgene encoded TGF-beta in islets prevents autoimmune diabetes in NOD mice by a local mechanism. J Autoimmun 2002;19:9–22.
145. Salomon B, Bluestone JA. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu Rev Immunol 2001;19:225–52.
146. Green EA, Eynon EE, Flavell RA. Local expression of TNFalpha in neonatal NOD mice promotes diabetes by enhancing presentation of islet antigens. Immunity 1998;9: 733–43.

 ISSN 0785-3890

147. Amrani A, Serra P, Yamanouchi J, Han B, Thiessen S, Verdaguer J, et al. CD154-dependent priming of diabetogenic CD4(+) T cells dissociated from activation of antigen-presenting cells. Immunity 2002;16:719–32.
148. Wong FS, Visintin I, Wen L, Granata J, Flavell R, Janeway CA. The role of lymphocyte subsets in accelerated diabetes in nonobese diabetic-rat insulin promoter-B7-1 (NOD-RIP-B7-1) mice. J Exp Med 1998;187:1985–93.
149. Yang XD, Tisch R, Singer SM, Cao ZA, Liblau RS, Schreiber RD, et al. Effect of tumor necrosis factor alpha on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. J Exp Med 1994;180:995–1004.
150. Green EA, Flavell RA. The temporal importance of TNFalpha expression in the development of diabetes. Immunity 2000;12:459–69.
151. Green EA, Choi Y, Flavell RA. Pancreatic lymph node-derived CD4(+)CD25(+) Treg cells: highly potent regulators of diabetes that require TRANCE-RANK signals. Immunity 2002;16:183–91.
152. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 2000;12:431–40.
153. Thompson CB, Allison JP. The emerging role of CTLA-4 as an immune attenuator. Immunity 1997;7:445–50.
154. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997; 389:737–42.
155. Letterio JJ, Roberts AB. Regulation of immune responses by TGF-beta. Annu Rev Immunol 1998;16:137–61.
156. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19:683–765.
157. Medzhitov R, Janeway CA, Jr. Decoding the patterns of self and nonself by the innate immune system. Science 2002;296: 298–300.
158. Garside P, Mowat AM. Mechanisms of oral tolerance. Crit Rev Immunol 1997;17:119–37.
159. Pape KA, Merica R, Mondino A, Khoruts A, Jenkins MK. Direct evidence that functionally impaired CD4+ T cells persist in vivo following induction of peripheral tolerance. J Immunol 1998;160:4719–29.
160. Appel H, Seth NP, Gauthier L, Wucherpfennig KW. Anergy induction by dimeric TCR ligands. J Immunol 2001;166:5279–85.
161. Casares S, Hurtado A, McEvoy RC, Sarukhan A, von Boehmer H, Brumeanu TD. Down-regulation of diabetogenic CD4+ T cells by a soluble dimeric peptide-MHC class II chimera. Nat Immunol 2002;3:383–91.
162. Hackstein H, Morelli AE, Thomson AW. Designer dendritic cells for tolerance induction:guided not misguided missiles. Trends Immunol 2001;22:437–42.
163. Ashkenazi A, Dixit VM. Apoptosis control by death and decoy receptors. Curr Opin Cell Biol 1999;11:255–60.
164. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000;192:1027–34.
165. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2001;2:261–8.
166. Min WP, Gorczynski R, Huang XY, Kushida M, Kim P, Obataki M, et al. Dendritic cells genetically engineered to express Fas ligand induce donor-specific hyporesponsiveness and prolong allograft survival. J Immunol 2000;164:161–7.
167. Matsue H, Matsue K, Walters M, Okumura K, Yagita H, Takashima A. Induction of antigen-specific immunosuppression by CD95L cDNA- transfected 'killer' dendritic cells. Nat Med 1999;5:930–7.
168. Buonocore S, Van Meirvenne S, Demoor FX, Paulart F, Thielemans K, Goldman M, et al. Dendritic cells transduced with viral interleukin 10 or Fas ligand: no evidence for induction of allotolerance in vivo. Transplantation 2002;73: S27–30.
169. Maksimow M, Santanen M, Jalkanen S, Hanninen A. Responding naive T cells differ in their sensitivity to Fas-engagement: early death of many T cells is compensated by costimulation of surviving T cells. Blood 2003:in press.
170. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. Cell 2001;106:255–8.
171. Falcone M, Lee J, Patstone G, Yeung B, Sarvetnick N. B lymphocytes are crucial antigen-presenting cells in the pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice. J Immunol 1998;161:1163–8.
172. King C, Davies J, Mueller R, Lee MS, Krahl T, Yeung B, et al. TGF-beta1 alters APC preference, polarizing islet antigen responses toward a Th2 phenotype. Immunity 1998;8:601–13.
173. Caux C, Massacrier C, Vanbervliet B, Dubois B, de Saint-Vis B, Dezutter-Dambuyant C, et al. CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. Adv Exp Med Biol 1997;417:21–5.
174. Markowicz S, Engleman EG. Granulocyte-macrophage colony-stimulating factor promotes differentiation and survival of human peripheral blood dendritic cells in vitro. J Clin Invest 1990;85:955–61.
175. Dhodapkar MV, Steinman RM, Sapp M, Desai H, Fossella C, Krasovsky J, et al. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. J Clin Invest 1999;104:173–80.
176. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med 2001;193:233–8.
177. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. Annu Rev Immunol 1997;15:617–48.
178. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993; 259:1745–9.
179. Karges W, Pechhold K, Al Dahouk S, Riegger I, Rief M, Wissmann A, et al. Induction of autoimmune diabetes through insulin (but not GAD65) DNA vaccination in nonobese diabetic and in RIP-B7.1 mice. Diabetes 2002;51: 3237–44.
180. Quintana FJ, Carmi P, Cohen IR. DNA vaccination with heat shock protein 60 inhibits cyclophosphamide-accelerated diabetes. J Immunol 2002;169:6030–5.
181. Quintana FJ, Rotem A, Carmi P, Cohen IR. Vaccination with empty plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity. J Immunol 2000;165:6148–55.
182. Tighe H, Corr M, Roman M, Raz E. Gene vaccination: plasmid DNA is more than just a blueprint. Immunol Today 1998;19:89–97.
183. Schirmbeck R, Reimann J. Revealing the potential of DNA-based vaccination: lessons learned from the hepatitis B virus surface antigen. Biol Chem 2001;382:543–52.
184. Stevenson FK, Rosenberg W. DNA vaccination: a potential weapon against infection and cancer. Vox Sang 2001;80:12–8.
185. Bonifaz L, Bonnyay D, Mahnke K, Rivera M, Nussenzweig MC, Steinman RM. Efficient targeting of protein antigen to

 ISSN 0785-3890

the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J Exp Med 2002;196:1627–38.

186. Burt RK, Slavin S, Burns WH, Marmont AM. Induction of tolerance in autoimmune diseases by hematopoietic stem cell transplantation: getting closer to a cure? Blood 2002;99:768–84.

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Diabetologia

Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity

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Summary Identification of islet autoantigens offers the possibility that antibody tests other than islet cell antibodies may be used for assessing risk of insulin-dependent diabetes mellitus (IDDM). The aim of this study was to determine the combination of islet autoantibody markers that could identify most future cases of IDDM. Islet cell antibodies, antibodies to glutamic acid decarboxylase $(GAD)_{65}$, 37,000/40,000 M_r islet tryptic fragments, carboxypeptidase-H, and islet cell autoantigen (ICA)69 were measured in sera from 100 newly-diagnosed IDDM patients, 27 individuals prior to onset of IDDM, and 83 control subjects. Islet cell antibodies were detected in 88% of IDDM patients and 81% with pre-IDDM, GAD_{65} antibodies in 70% of IDDM patients and 89% with pre-IDDM, and antibodies to 37,000/40,000 M_r islet tryptic fragments in 54% of IDDM patients and in 48% with pre-IDDM. The latter were found only in conjunction with islet cell antibodies and were more frequent in young onset cases. All 20 IDDM patients and the 3 pre-IDDM subjects who had islet cell antibodies without GAD_{65} antibodies had antibodies to 37,000/40,000 M_r islet tryptic fragments, and all but one had disease onset before age 15 years. No sera strongly immunoprecipitated in vitro translated ICA69 or carboxypeptidase-H; 4% of patients had anti-ICA69 and 11% anti-carboxypeptidase-H levels above those of the control subjects. The findings suggest that none of the single antibody specificities are as sensitive as islet cell antibodies, but that a combination of GAD_{65} antibodies and antibodies to 37,000/40,000 M_r islet tryptic fragments has the potential to identify more than 90% of future cases of IDDM. Such a strategy could eventually replace islet cell antibodies in population screening for IDDM risk assessment. [Diabetologia (1995) 38: 816–822]

Key words Islet cell antibodies, glutamic acid decarboxylase$_{65}$ antibodies, islet autoantigens, insulin-dependent diabetes mellitus prediction, carboxypeptidase-H, ICA69.

Treatments that may delay or prevent the onset of insulin-dependent diabetes (IDDM) are already being tested in large-scale trials in family members of children with the disease [1]. Although current risk assessment strategies are based on prospective family studies [2–4], only around 10% of patients have a first-degree family history of IDDM. If preventive therapies are to have a real impact on the frequency of disease, they will eventually have to be evaluated and applied in the population as a whole.

Islet cell antibodies (ICA) are the most widely used antibody marker in risk assessment [5]. Over 80% of first-degree relatives who develop IDDM have detectable ICA, and unaffected relatives with ICA are at greatly increased risk of developing the disease [2–4]. The technical limitation of ICA measurements is that they rely on semi-quantitative indi-

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Abbreviations: CL, Confidence limits; CPH, carboxypeptidase-H; GAD, glutamic acid decarboxylase; GADA, glutamic acid decarboxylase$_{65}$ antibodies; IAA, insulin autoantibodies; ICA, islet cell antibodies; IDDM, insulin-dependent diabetes mellitus; JDF units, Juvenile Foundation Diabetes units.

rect immunofluorescent assays [6] which, despite much improvement [7], are difficult to standardize [8, 9]. Antibodies to the islet autoantigen glutamic acid decarboxylase (GAD) are also detected prior to onset of IDDM [10–14]. The recent development of GAD antibody (GADA) assays [15, 16] which use small volumes of serum and detect antibodies in over 70 % of IDDM patients, now offer the possibility to screen large sample numbers. Similar methods could potentially be applied to other proposed antibody markers [17] and it may ultimately be possible to develop a single assay measuring antibodies to several islet antigens, thus replacing ICA testing. .

We have measured antibodies to GAD, 37,000/40,000 M_r islet tryptic fragments (anti-37 K) [18], carboxypeptidase-H (CPH) [19], and ICA69 [20], in 100 consecutive patients with newly-diagnosed IDDM, in 83 control subjects and in samples taken prior to onset of IDDM from 27 individuals in the Barts-Windsor [2] and Barts-Oxford family studies [13]. The aim of the study was to select the best combination of antibody markers for use in a screening test for IDDM risk assessment and to evaluate these in comparison with ICA.

Subjects, materials and methods

Subjects

Patients with IDDM. Serum samples were obtained at onset of disease from 100 consecutive patients with IDDM diagnosed at the San Raffaele Hospital, Milan, Italy, during 1990 and 1991. IDDM was defined on the basis of a clinical diagnosis. All patients started insulin at the time of diagnosis and remained insulin-dependent thereafter. Patients had a median age of 11 years (range 1 to 40 years). Sixty-nine were diagnosed before age 15 years, and 53 were male.

Pre-IDDM. Serum taken prior to diagnosis was available in 27 of the 29 family members who have developed IDDM whilst under follow-up in the Barts-Windsor [2] and Barts-Oxford family studies in the UK [13]. They were all included irrespective of ICA status. The first available serum sample from each individual was tested. Six parents and 21 siblings of the diabetic proband were studied. The median age at sample collection was 17 years (range 2 to 55 years) and at diagnosis of IDDM was 20 years (range 3 to 57 years). The median time to diagnosis was 2.7 years (range 0.2 to 8.1 years).

Control subjects. Samples were collected from 83 normal children and blood donors in the Milan area. They had a median age of 12 years (range 1 to 40 years), 37 were male.

Recombinant autoantigens

Poly-A RNA was isolated from 20,000 purified human islets using Dynabeads Oligo dT(25) (Dynal, Oslo, Norway) [21]. Reverse transcription of 3 µl of the poly-A was performed using a mixture of random hexamers (Perkin Elmer Cetus, Norwalk, Conn., USA) to obtain cDNA [22]. The full length coding sequences of GAD_{65} and ICA69 were obtained after polymerase chain reaction amplification [23] of cDNA. The amplified products were ligated into the plasmid vector pCRII (Invitrogen, San Diego, Calif., USA), and then subcloned into the ECoR1 (GAD_{65}) or BamH1 (ICA69) cloning sites of the pGEM 3 vector (Promega, Madison, Wis., USA) under the control of the SP6 promotor for in vitro transcription and expression [24]. Recombinant rat CPH cDNA, cloned into the pSP64 vector (Promega) under the control of the SP6 promotor, was kindly provided by Dr. J. Hutton, Addenbrooke's Hospital, Cambridge, UK.

Radiolabelled recombinant proteins were obtained from in vitro coupled transcription and translation of 1 µg of the appropriate plasmid by SP6 RNA polymerase and rabbit reticulocyte lysate (Promega) in the presence of 40 µCi ^{35}S-methionine (Amersham International, Amersham Bucks., UK). Unincorporated label was removed by gel chromatography on a NAP 5 column (Pharmacia, Uppsala, Sweden).

Autoantibody measurements

GADA. Antibodies to in vitro translated GAD_{65} were measured using a method similar to that previously described [16]. A total of 15,000 cpm of labelled GAD_{65} protein diluted in 50 mmol/l Tris-HCl pH 7.2, 150 mmol/l NaCl, and 1 % Tween 20 (TBST) was added to 2 µl of serum to a final volume of 50 µl in 96-deep well microtitre plates and incubated overnight on ice. Immunocomplexes were isolated on 1 mg protein A Sepharose (Pharmacia), pre-swelled and resuspended in 50 µl of TBST, followed by incubation for 1 h at 4 °C with shaking. The immunocomplexes bound to protein A were washed once with 750 µl of cold TBST and transferred to a 96-well filtration system (Millipore, Bedford, Mass., USA) with a 0.45-µm filter at the bottom of the wells. The unit was placed on a vacuum device and after 10 washes, each of 150 µl of TBST, the bottom of each well was punched into a scintillation vial containing 2.5 ml of scintillation fluid (Ultimagold, Packard, Groningen, The Netherlands), and cpm measured in a scintillation counter (Kontron Instruments, Montigny le Bretonnieux, France). All samples were tested in duplicate. Results are expressed as mean cpm. The interassay coefficient of variation on 10 assays was less than 15 % for control samples with mean cpms of 300 and 600. This assay detected none of 32 control samples and 32 of 39 (82 %) IDDM samples in the 2nd International GAD antibody workshop.

Anti-carboxypeptidase-H and anti-ICA69. Antibodies to in vitro translated CPH and ICA69 were measured as previously described for anti-ICA69 [24]. A total of 17,000 cpm of labelled protein diluted in TBST was added to 4 µl of serum in a final volume of 50 µl in 96-deep-well microtitre plates and incubated overnight on ice. Immunocomplexes were isolated on 2 mg protein A Sepharose (Pharmacia), pre-swelled and resuspended in 50 µl of TBST, followed by incubation for 1 h at 4 °C with shaking. For anti-CPH, immunocomplexes bound to protein A were washed eight times, each with 750 µl of cold TBST, transferred to scintillation vials containing 2.5 ml of scintillation fluid, and the cpm measured. For anti-ICA69, immunocomplexes bound to protein A were washed twice, each with 750 µl of cold TBST and transferred to a 96-well filtration system, washed and counted as for GADA. Rabbit polyclonal anti-CPH provided by Dr. J. Hutton and a rabbit polyclonal antiserum raised against purified recombinant ICA69 protein (Primm, Milan, Italy) strongly immunoprecipitated the in vitro translated proteins (Fig. 1).

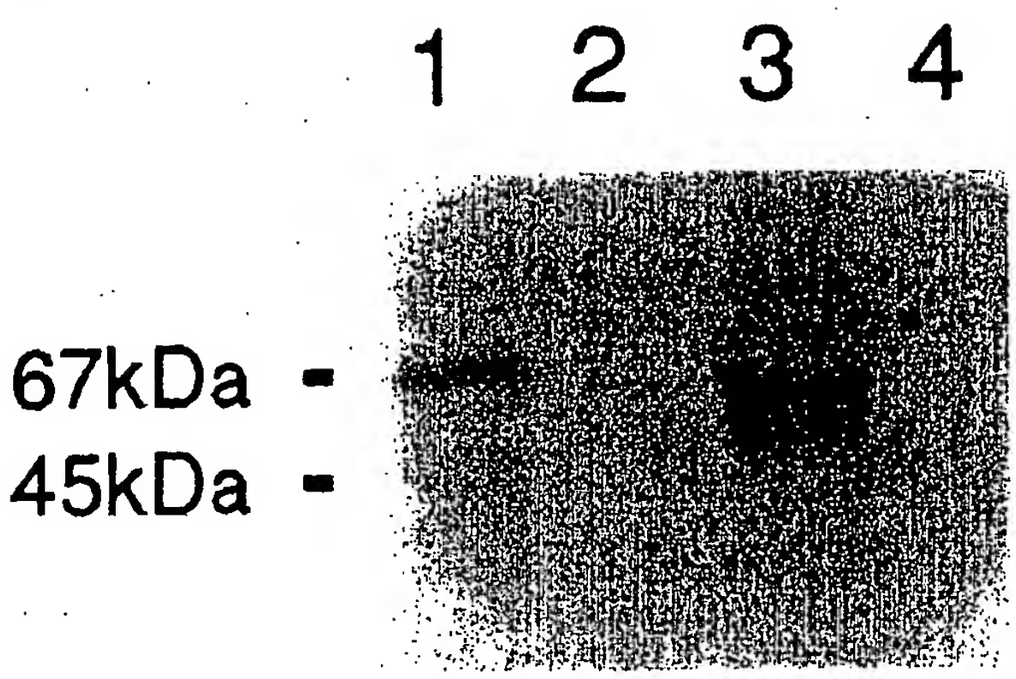

Fig.1. Immunoprecipitation of in vitro translated, ICA69 (lanes 1 and 2) and CPH (lanes 3 and 4). ^{35}S methionine labelled protein was immunoprecipitated with 0.2 µl rabbit anti-ICA69 (lane 1), 0.2 µl rabbit anti-CPH (lane 3), and 2 µl preimmune rabbit sera (lanes 2 and 4), and run on 10 % polyacrylamide gel electrophoresis under denaturing conditions

Dilutions of these antisera were used as controls in each assay.

Anti-37 K. Rat insulinoma cell line (RIN 5AH) was maintained in tissue culture in RPMI 1640 medium containing 10 % fetal bovine serum (Sigma, St. Louis, Mo., USA). Subconfluent RIN cells in a 162-cm^2 tissue culture flask (approximately 1×10^8 cells) were radiolabelled in methionine-free RPMI 1640 medium (Sigma) with 1 mCi of [^{35}S] in vitro cell labelling mix (Promix – Amersham International) for 5 h at 37 °C; the cell pellet was then frozen at –80 °C until processed. For cell lysis, 400 µl of 10 mmol/l Hepes (pH 7.4), 150 mmol/l NaCl, 0.1 % (weight/volume) aprotinin, and 2 % Triton X-114 were added to a pellet of radiolabelled insulinoma cells followed by 2 h incubation at 4 °C with agitation. The homogenate was centrifuged at 10,000 *g* for 15 min at 4 °C, and the supernatant collected and incubated at 30 °C for phase separation. Triton X-114 detergent phase was precleared with 50 µl of normal human serum for 18 h at 4 °C followed by binding to 100 µl of packed protein A Sepharose (Sigma) for 30 min at 4 °C. Aliquots of 20 µl extract containing 4×10^6 cpm of radiolabelled protein were incubated with 5 µl of test serum for 5 h at 4 °C. Immune complexes were isolated on 20 µl of packed protein A Sepharose, washed three times with 1 ml 20 mmol/l Hepes (pH 7.4), 500 mmol/l NaCl, and once with 1 ml of water. They were then incubated with 50 µl of 0.1 mg/ml trypsin in 10 mmol/l Hepes (pH 7.4), and 150 mmol/l NaCl for 20 min at 4 °C, washed with 1 ml of water before processing for SDS-10 % polyacrylamide gel electrophoresis and autoradiography. Serum samples were regarded as anti-37 K positive if 37kDa and/or 40kDa polypeptide bands were detected on the autoradiogram.

Islet cell antibodies (ICA). These were measured in undiluted sera by indirect immunofluorescence on 4-µm cryostat sections of blood group O human pancreas as previously described [2]. Positive samples were titrated to end point in doubling dilutions in 10 mmol/l phosphate-buffered saline (pH 7.2). Local (Milan) standard sera calibrated to 2.5, 5, 10, 20, 40 and 80 Juvenile Diabetes Foundation (JDF) units were included in each assay. End-point titres of test samples were converted to JDF units by comparison with a standard curve of $\log_2$ JDF units vs $\log_2$ of end-point titre of the standard sera. The threshold of ICA detection was 2 JDF units.

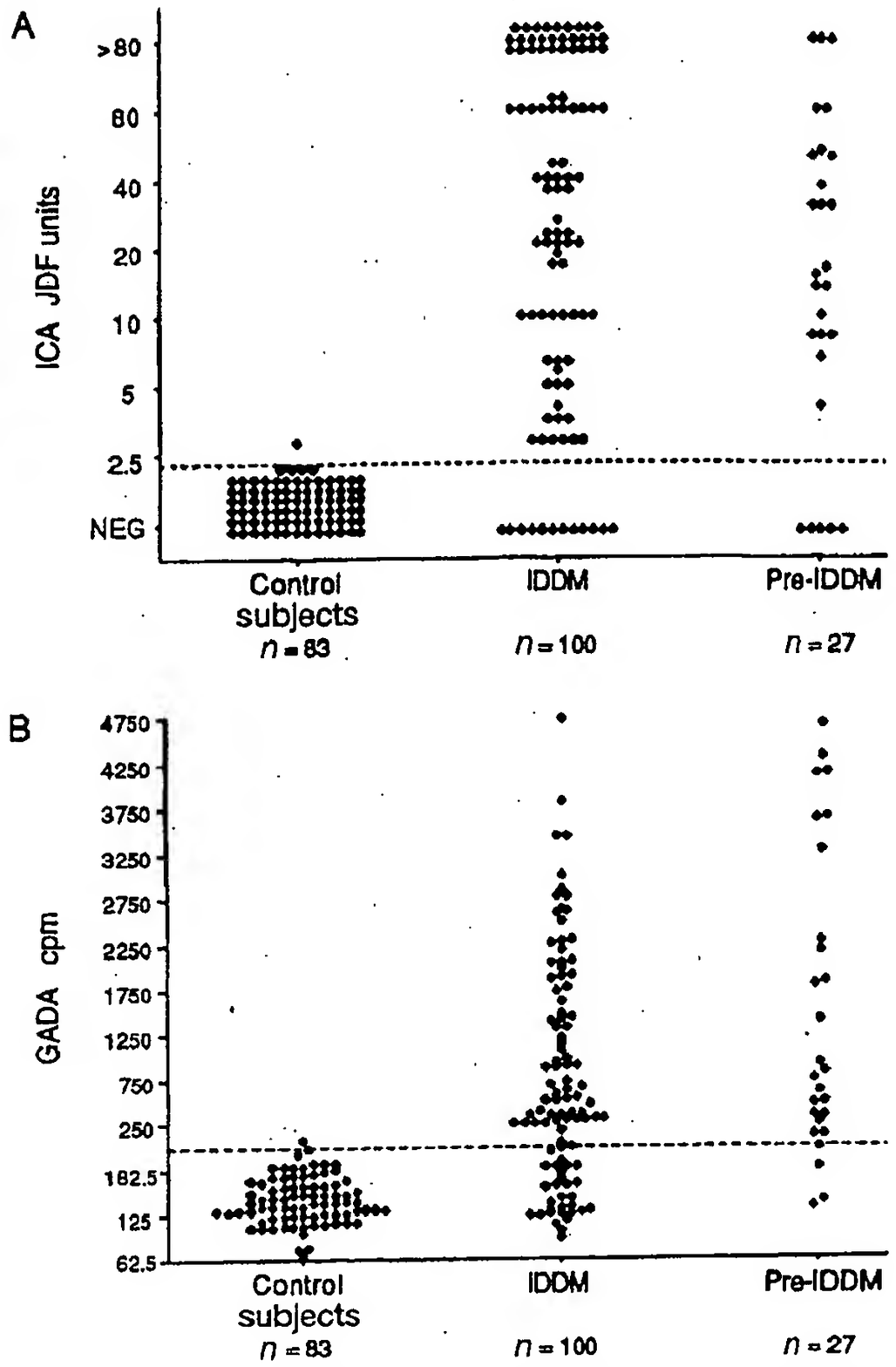

Fig.2. (A) ICA and (B) GADA distribution in study groups. The upper first percentile of control subjects is shown by the broken lines

Insulin autoantibodies (IAA). These were measured on samples in which ICA were not detected using a radio-binding assay as previously described [25].

Statistical analysis

The threshold for positivity was selected as the upper first percentile of control results in each assay. All assays will have the same specificity (99 %) allowing comparison of sensitivities between them. All comparisons were made using the chi-square test with Yates' correction. *p* values for comparisons between paediatric (onset < 15 years) and older-onset cases were corrected for the number of categories ($n = 5$). Where appropriate, 95 % confidence limits (CL) are given.

Results

Insulin-dependent diabetes mellitus (IDDM). ICA were detected in 88 of 100 cases at onset of IDDM

Table 1 Islet antibody prevalence

Patient group	Age (years)	*n*	ICA	GADA	Anti-37K	Anti-CPH	Anti-ICA69
IDDM							
	≤ 15	69	64 (93 %)	45 (65 %)	43 (62 %)	10 (13 %)	2 (3 %)
	> 15	31	24 (77 %)	25 (81 %)	11 (35%)[a]	3 (10 %)	0 (0 %)
Total		100	88	70	54	13	2
Pre-IDDM							
	≤ 15	10	9 (90 %)	8 (80 %)	5 (50 %)	1 (10 %)	1 (10 %)
	> 15	17	13 (76 %)	16 (94 %)	8 (46 %)	0	2 (12 %)
Total		27	22 (81 %)	24 (89 %)	13 (48 %)	1 (4 %)	3 (11 %)
Control subjects		83	1 (1 %)	1 (1 %)	0 (0 %)	1 (1 %)	1 (1 %)

[a] $p < 0.05$ vs ≤ 15 years

Table 2. Islet antibody combination

Patient group	Age (years)	*n*	ICA/GADA/Anti-37K				
			+/+/+	+/+/–	+/–/+	–/+/–	–/–/–
IDDM							
	≤ 15	69	23 (33 %)	21 (30 %)	20 (29 %)	1 (1 %)	4 (6 %)
	> 15	31	11 (35 %)	13 (40 %)	0 (0%)[a]	1 (3 %)	6 (19 %)
Total		100	34	34	20	2	10
Pre-IDDM							
	≤ 15	10	3 (30 %)	4 (40 %)	2 (20 %)	1 (10 %)	0
	> 15	17	7 (41 %)	5 (29 %)	1 (6 %)	4 (24 %)	0
Total		27	10 (37 %)	9 (33 %)	3 (11 %)	5 (19 %)	0
Control subjects		83	0 (0 %)	1 (1 %)	0 (0 %)	0 (0 %)	82 (99 %)

[a] $p < 0.02$ vs ≤ 15 years

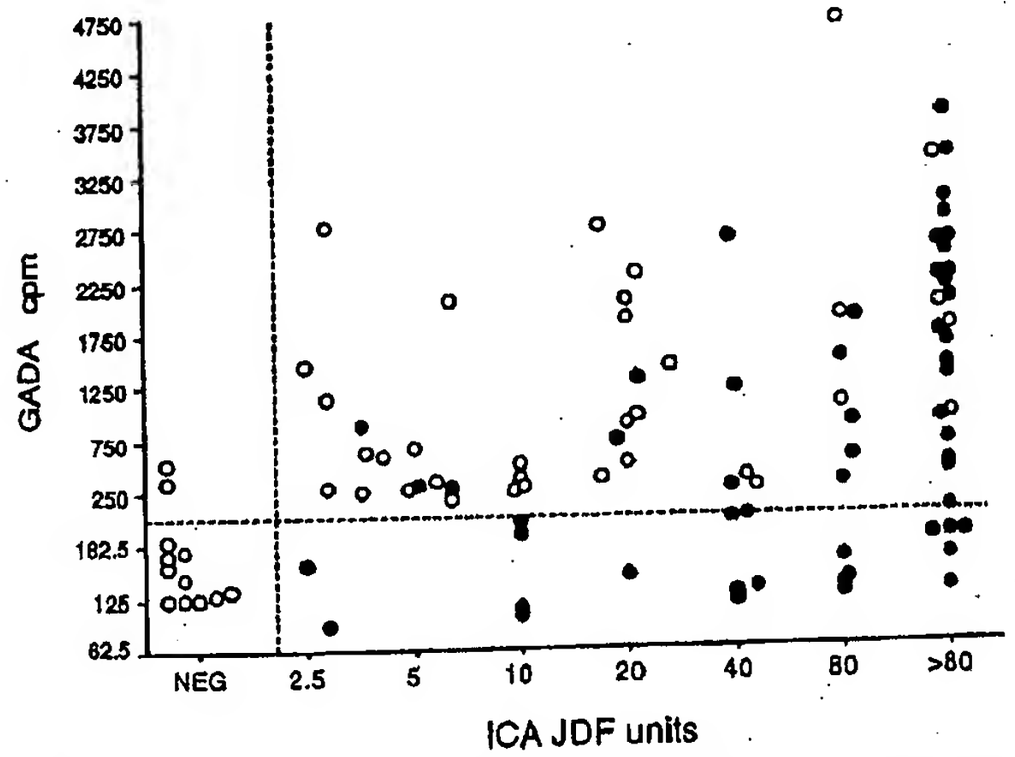

Fig. 3. Islet autoantibodies in sera from 100 newly-diagnosed IDDM patients. The filled symbols represent those in which anti-37 K were detected. The upper first percentile of 83 control sera are represented by the broken lines at 2 JDF units for ICA and 217 cpm for GADA

(CL: 80–94 %); 27 of these had between 2 and 19 JDF units and 61 ≥ 20 JDF units (Fig. 2A). ICA were the most frequently detected single humoral marker ($p < 0.01$ vs GADA; $p < 10^{-6}$ vs anti-37 K) (Table 1).

GADA were found in 70 IDDM patients (CL: 60–79 %) (Table 1; Fig. 2B), including 2 of the 12 patients without detectable ICA. ICA in the absence of GADA were found in 20 patients, all of whom had an age of onset before age 15 years ($p < 0.02$ vs > 15 years) (Table 2). There was no correlation between GADA levels and ICA titres (Fig. 3).

Anti-37 K were found in 54 patients (CL: 44–64 %); all had ICA ($p < 0.001$ vs ICA negative). They were detected more frequently in patients with disease onset before age 15 years ($p < 0.05$). Anti-37 K were strongly associated with high titre ICA and were detected in 35 of 42 (81 %) of those with ICA greater than 40 JDF units ($p < 0.002$). All 20 cases with ICA in the absence of GADA had 37 K antibodies (Table 2). Anti-37 K were not associated with the presence of GADA (Fig. 3).

All sera were tested for antibodies to CPH and ICA69. None strongly immunoprecipitated in vitro translated CPH or ICA69. Thirteen had anti-CPH levels and four anti-ICA69 levels above the upper first percentile of normal control subjects (Fig. 4).

ICA and/or GADA were detected in 90 % of cases (CL: 82–95 %). The same cases could also be identified by the presence of GADA and/or anti-37 K. Four (6 %) patients with onset before age 15 years and 6 (19 %) patients with onset after age 15 years

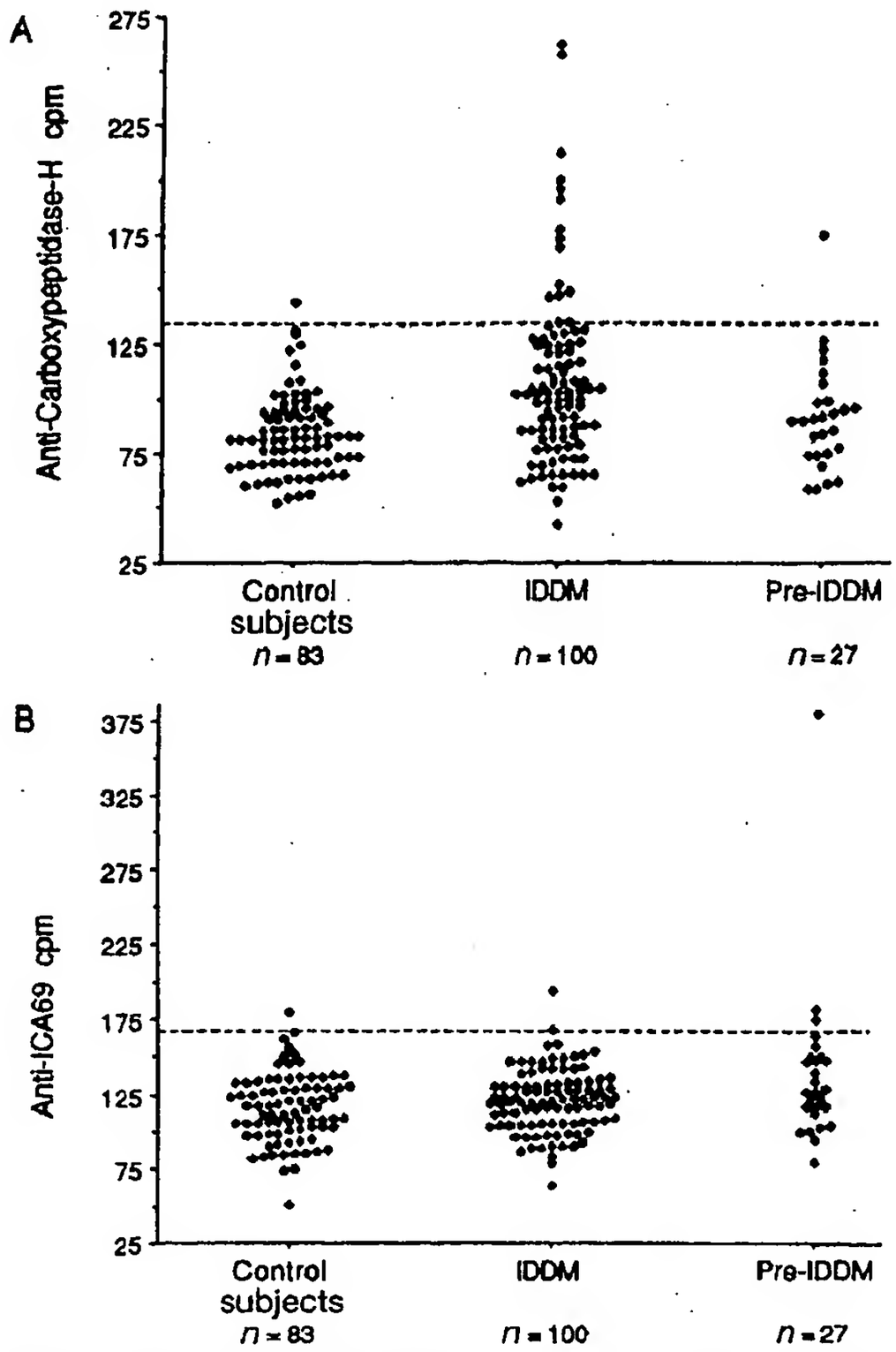

Fig. 4 (A, B). Immunoprecipitation of in vitro translated (**A**) CPH and (**B**) ICA69. The upper first percentile of control subjects is shown by the broken lines

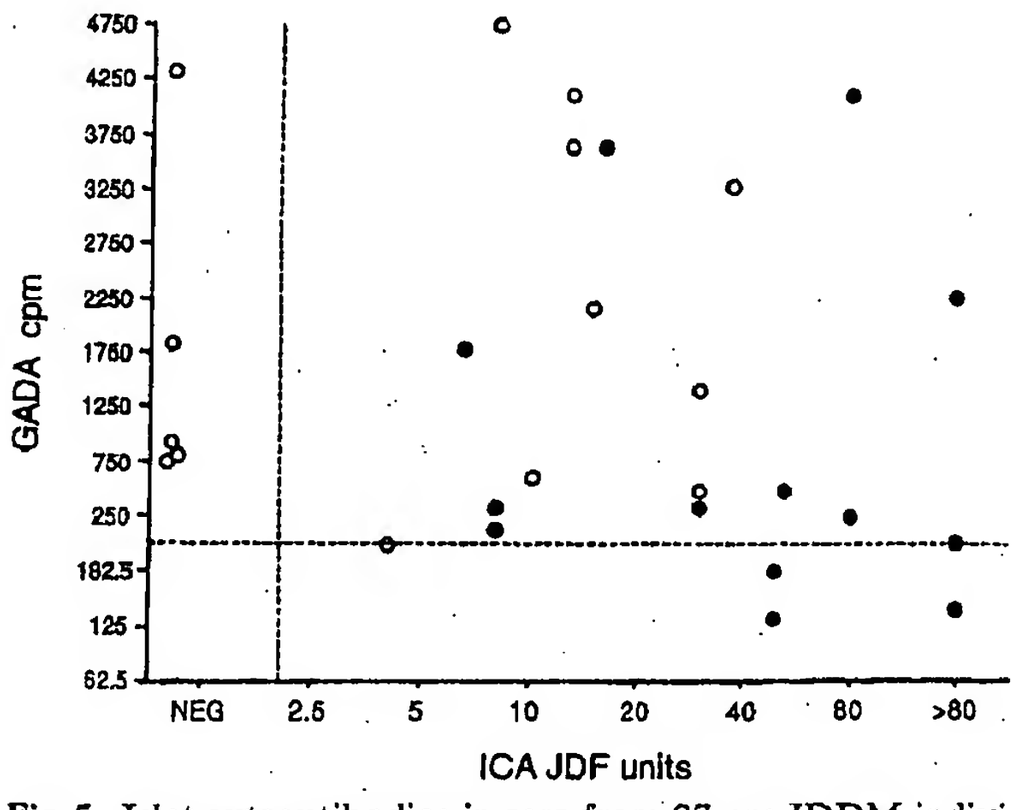

Fig. 5. Islet autoantibodies in sera from 27 pre-IDDM individuals. The filled symbols represent those in which anti-37 K were detected. The upper first percentile of 83 control sera are represented by the broken lines at 2 JDF units for ICA and 217 cpm for GADA

were negative for all three markers (Table 2). One of the antibody negative cases had IAA; none had increased levels of anti-ICA69 or anti-CPH.

Pre-IDDM. ICA and GADA were the most frequently detected single markers prior to onset of IDDM (Table 1; Fig. 5). Together they identified all 27 cases (CL: 87–100 %). Five (19 %) had GADA in the absence of ICA and three (11 %) had ICA in the absence of GADA. Anti-37 K were found in 13 cases (48 %; CL: 29–68 %), all of whom also had ICA. These included all three cases with ICA in the absence of GADA (Table 2). One (4 %) had increased levels of anti-CPH, and three (11 %) had anti-ICA69 levels above the upper first percentile of normal control subjects (Fig. 4). No significant differences between pre-IDDM and IDDM at onset were found.

Discussion

Our aim was to develop a screening method based on islet autoantibody markers that will potentially identify most future cases of IDDM. The most sensitive markers were ICA and GADA. Both were detected in 68 % of IDDM and 70 % of pre-IDDM sera, and 90 % of all IDDM and pre-IDDM sera had at least one of these markers. The majority of IDDM cases in which neither ICA nor GADA were detected were adult so that 94 % of cases with onset before age 15 had at least one of these markers as against 81 % over that age. Despite the high sensitivity of a combination of ICA and GADA, a significant proportion of patients had only one of these markers. In particular, 28 % of those with childhood-onset disease had ICA without GADA.

Anti-37 K were the next most prevalent marker in newly-diagnosed IDDM. They were found only in conjunction with ICA, and strikingly, in all cases with ICA in the absence of GADA. Here again, 90 % of IDDM cases, including 95 % of those with onset before age 15, and all pre-IDDM sera had either GADA or anti-37 K. ICA69 and CPH antibodies measured by immunoprecipitation were not detected in any of the GADA/anti-37 K negative cases, and did not increase sensitivity. IAA identified only one of these cases.

These findings suggest that ICA are the most sensitive screening test, particularly in childhood-onset diabetes, provided an assay is used that can detect low levels of antibody reproducibly. Even so, the assay is difficult to standardize, and many laboratories are unable to measure low levels consistently [7, 9]. ICA contain more than one specificity [26–29], not all of which appear to be associated with IDDM [27]. Furthermore, ICA in the absence of other islet antibody specificities are only weakly predictive of IDDM in first-degree relatives [13] or schoolchildren

[14]. In both groups increased risk is concentrated in the minority in whom more than one islet autoantibody specificity is present. In the present study, ICA were always found in association with either GADA or anti-37 K in sera taken both at and prior to diagnosis. It may therefore be preferable to undertake initial screening with those antibody specificities associated with ICA that are more closely related to disease.

GADA measurement has been proposed as a test to assess IDDM risk [12]. The assay output is numerical, removing some observer bias, and since many samples with low ICA levels have GADA levels well above the threshold for positivity used in this study, their use would avoid the difficulty of measuring low titre ICA. GADA appear to be as sensitive as ICA in adult-onset IDDM, but we found that 28 % of patients diagnosed before age 15 years have ICA in the absence of GADA at or before disease onset. This group is of particular importance in that every case had anti-37 K, a marker associated with rapid progression to insulin-requiring diabetes [13, 14, 30], thus implying an aggressive disease process. GADA measurement alone is therefore unlikely to provide as sensitive a screening test as ICA.

Our findings suggest that measuring GADA and anti-37 K could provide a test that would retain the sensitivity of ICA. Combining anti-37 K and GADA significantly enhances the sensitivity of GADA alone, and the combination of anti-37 K and ICA identifies a subgroup with markedly increased risk [13, 14, 30]. Current methods for anti-37 K are laborious and costly, and preclude the general application of this marker. Anti-37 K are however, like GADA, detected by immunoprecipitation of liquid phase antigen using small serum volumes. Therefore, provided the 37k antigen is cloned, it is likely that assays can be developed which will measure antibodies to in vitro translated recombinant GAD and 37 K antigen simultaneously. Additions of ICA69 or CPH antibodies contributed little to the analysis. Antibodies to ICA69 which have been detected in solid-phase assays [20, 24] rarely immunoprecipitate in vitro translated antigen [24], and increased binding to CPH was detected in only few sera and was not confined to GADA negative cases. IAA were not tested in all cases used in the present analysis, but we have previously found them to be absent prior to disease onset in a number of those with anti-37 K in the absence of GADA [13, 14]. Furthermore, the large serum volumes currently needed for the detection of IAA render these unsuitable for adaptation to screening assays for use in the general population.

In conclusion, we propose that a combination of GADA and anti-37 K testing could eventually replace ICA in screening for risk of IDDM. Until assays with the capacity to measure anti-37 K in large numbers of samples are available, we suggest that maximum sensitivity will be achieved by a combination of GADA and ICA. The screening strategies we propose have the potential to identify more than 90 % of future cases. We have tested this prospectively only in first degree relatives of children with IDDM in the UK, but the combinations of antibodies seen in these individuals are not different to those in the Italian non-familial cases at diagnosis, or to the few cases identified in a prospectively followed UK population of school children [14]. It is important to emphasise that the aim of the present analysis is to identify an approach to screening that maximizes sensitivity, thus identifying the greatest possible number of individuals at risk of developing IDDM within the general population. Additional or sequential tests, for example genetic analysis or metabolic testing, may then need to be applied to those with antibody markers in order to improve the specificity of prediction [31]. This approach promises in time to identify levels of risk sufficient to justify intervention trials in those with no family history of IDDM.

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References

1. Skyler JS, Marks JB (1993) Immune intervention in type 1 diabetes mellitus. Diabetes Rev 1: 15–42
2. Bonifacio E, Bingley PG, Shattock M et al. (1990) Quantification of islet cell antibodies and prediction of insulin-dependent diabetes. Lancet 335: 147–149
3. Riley WJ, Maclaren NK, Krischer J et al. (1990) A prospective study of the development of diabetes in relatives of patients with insulin-dependent diabetes. N Engl J Med 323: 1167–1172
4. Knip M, Vahasalo P, Karjalainen J, Lounamaa R, Akerblom HK, and the Childhood Diabetes in Finland Study group (1994) Natural history of preclinical IDDM in high risk siblings. Diabetologia 37: 388–393
5. Bottazzo GF, Florin-Christensen A, Doniach D (1974) Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiency. Lancet ii:1279–1283
6. Gleichmann H, Bottazzo GF (1987) Progress towards standardization of cytoplasmic islet cell antibody assays. Diabetes 36: 578–584
7. Bonifacio E, Lernmark A, Dawkins RL et al. (1988) Serum exchange and use of dilutions have improved precision of measurement of islet cell antibodies. J Immunol Methods 106: 83–88
8. Bonifacio E, Boitard C, Gleichmann H, Shattock MA, Molenaar JL, Bottazzo GF (1990) Assessment of precision, concordance, specificity and sensitivity of islet cell antibody measurement in 41 assays. Diabetologia 33: 731–736
9. Greenbaum CJ, Palmer JP, Nagataki S et al. (1992) Improved specificity of ICA assays in the Fourth Internation-

al Immunology of Diabetes serum exchange Workshop. Diabetes 41: 1570–1574
10. De Aizpurua HJ, Harrison LC, Cram DS (1992) An ELISA for antibodies to recombinant glutamic acid decarboxylase in IDDM. Diabetes 41: 1182–1187
11. Thivolet CH, Tappaz M, Durand A et al. (1992) Glutamic acid decarboxylase (GAD) autoantibodies are additional predictive markers of type I (insulin-dependent) diabetes mellitus in high risk individuals. Diabetologia 35: 570–576
12. Tuomilehto J, Zimmet P, Mackay IR et al. (1994) Antibodies to glutamic acid decarboxylase as predictiors of insulin-dependent diabetes mellitus before clinical onset of disease. Lancet 343: 1383–1385
13. Bingley PJ, Christie MR, Bonifacio E et al. (1994) Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody positive relatives. Diabetes 43: 1304–1310
14. Genovese S, Bingley PJ, Bonifacio E et al. (1994) Combined analysis of IDDM-related autoantibodies in healthy schoolchildren. Lancet 344: 756
15. Grubin CE, Daniels T, Toivola B et al. (1994) A novel radiobinding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. Diabetologia 37: 344–350
16. Petersen JS, Hejnaes KR, Moody A et al. (1994) Detection of GAD_{65} antibodies in diabetes and other autoimmune diseases using a simple radioligand assay. Diabetes 43: 459–467
17. Bosi E, Bonifacio E (1994) Autoantibodies in insulin-dependent diabetes mellitus. J Endocrinol Invest 17: 521–531
18. Christie MR, Vohra G, Champagne P, Daneman D, Delovitch TL (1990) Distinct antibody specificities to a 64-kD islet cell antigen in type 1 diabetes as revealed by trypsin treatment. J Exp Med 172: 789–795
19. Castano L, Russo E, Zhou L, Lipes MA, Eisenbarth GS (1991) Identification and cloning of a granule autoantigen (carboxypeptidase-H) associated with type 1 diabetes. J Clin Endocrinol Metab 73: 1197–1201
20. Pietropaolo M, Castano L, Babu S et al. (1993) Islet cell autoantigen 69 kD (ICA69). Molecular cloning and characterization of a novel diabetes-associated autoantigen. J Clin Invest 92: 359–371
21. Hornes E, Jacobsen KS, Gabrielsen OS, Kornses LS, Jansen EB, Eselund M (1991) Purification of mRNA and DNA binding proteins using magnetic beads. In: Kemshead JT (ed) Magnetic separation techniques applied to cellular and molecular biology. Wordsmith Conference Publications. Somerset, UK, pp 197–205
22. Wang AM, Doyle MV, Mark FF (1989) Quantitation of mRNA by the polymerase chain reaction. Proc Natl Acad Sci USA 86: 9717–9721
23. Saiki, RK, Gelfand DH, Stoffel S et al. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487–491
24. Lampasona V, Ferrari M, Bosi E, Pastore RM, Bingley PJ, Bonifacio E (1994) Sera from patients with IDDM and healthy individuals have antibodies to ICA69 on Western blots but do not immunoprecipitate liquid phase antigen. J Autoimmun 7: 665–674
25. Levi-Marchal C, Bridel M-P, Sodoyez-Goffaux F et al. (1991) Superiority of radiobinding assay over ELISA for detection of IAAs in newly diagnosed type 1 diabetic children. Diabetes Care 14: 61–63
26. Genovese S, Bonifacio E, McNally JM et al. (1992) Distinct cytoplasmic islet cell antibodies with different risks for type 1 (insulin-dependent) diabetes mellitus. Diabetologia 35: 385–388
27. Gianani R, Pugliese A, Bonner-Weir S et al. (1992) Prognostically significant heterogeneity of cytoplasmic islet cell antibodies in relatives of patients with type I diabetes. Diabetes 41: 347–353
28. Timsit J, Caillat-Zucman S, Blondel H, Chédin P, Bach JF, Boitard C (1992) Islet cell antibody heterogeneity among type 1 (insulin-dependent) diabetic patients. Diabetologia 35: 792–795
29. Atkinson MA, Kaufman DL, Newman D, Tobin AJ, Maclaren NK (1993) Islet cell cytoplasmic autoantibody reactivity to glutamate decarboxylase in insulin-dependent diabetes. J Clin Invest 91: 350–356
30. Christie MR, Genovese S, Cassidy D et al. (1994) Antibodies to islet 37k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity. Diabetes 43: 1254–1259
31. Bingley PJ, Bonifacio E, Gale EAM (1993) Can we really predict IDDM? Diabetes 42: 213–220

1: Korean J Intern Med. 1995 Jan;10(1):1-9.

Relationships among 64k autoantibodies, pancreatic beta-cell function, HLA-DR antigens and HLA-DQ genes in patients with insulin-dependent diabetes mellitus in Korea.

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OBJECTIVES: Among autoantibodies detected in patients with insulin-dependent diabetes mellitus (IDDM), antibodies to 64,000(Mr) islet protein (64k), now recognized as glutamic acid decarboxylase(GAD), appear to be a even more predictive marker of IDDM than islet cytoplasmic antibody (ICA) or insulin autoantibody (IAA). We examined the relationships among 64k autoantibodies, pancreatic beta-cell function, HLA-DR antigens and HLA-D genes in patients with IDDM in Korea. METHODS: To identify the 64k autoantibody, the immunoprecipitation method was performed for 35 patient with IDDM and 10 normal controls. In patients with IDDM, serum C-peptide levels were measured and HLA-DR typings and HLA-DQA1 and DQB1 gen typings were performed. RESULTS: 12 of 35 (34%) patients with IDDM we positive for 64k autoantibody in contrast to none of 10(0%) normal controls. There were no differences in residual pancreatic beta-cell function between 6 autoantibody positive and negative groups. 64k autoantibody was detected more frequently in patients with recent (duration < 6 months, 10/25[40%]) a young -aged(aged < 15 years, 7/18[39%]) onset of IDDM. All of 3(100%) patients with HLA-DR3/DR4 heterotypes were positive in 64k autoantibody, contrast to 1 of 7(14%) patients without HLA-DR3 nor DR4. The frequencie of HLA-DQA1*0301, HLA-DQB1*0201, DQB1* 0302 and DQB1*0303 ge types were higher in patients with 64k autoantibody (12/12 [100%]) vs. with 64k autoantibody 18/22[81%], 5/11[45%] vs. without 64k autoantibody 5/22 [23%], 5/11[45%] vs. without 64k autoantibody 8/22[36%] and 6/11 [55%] v without 64k autoantibody 9/22[41%]. CONCLUSIONS: There results sugge that 64k autoantibodies have some relationship with HLA-DR, DQA1 and DQB1 genes, but not with residual pancreatic beta-cell function in Korean patients with IDDM.

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Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives.

by Polly J. Bingley, Michael R. Christie, Ezio Bonifacio, Ricardo Bonfanti, Marion Shattock, Maria-Teresa Fonte, Gian-Franco Bottazzo and Edwin A.M. Gale

Prediction of insulin-dependent diabetes mellitus (IDDM) is still largely based on islet cell antibodies (ICAs), but it may be improved by combined analysis with other humoral markers. We examined autoantibodies to insulin (IAAs), glutamic acid decarboxylase (GAD), and [M.sub.r] 37,000 and [M.sub.r] 40,000 fragments of islet antigens (37 and 40 kDa) together with ICA subtypes in 101 family members with ICAs [greater than or equal to]10 Juvenile Diabetes Foundation units (JDF U) followed for up to 14 years, of whom 18 have developed IDDM. Life-table analysis showed a 43% risk of IDDM within 10 years for those with ICAs [greater than or equal to]10 JDF U, rising to 53% for those with ICAs [greater than or equal to]20 JDF U. The risk for ICAs [greater than or equal to]10 JDF U was 62% in the family members in the youngest age quartile (<13.2 years) and fell with increasing age to 4% in those >40.7 years of age (P = 0.03). ICAs [greater than or equal to]10 JDF U combined with IAAs gave a risk of 84% (P = 0.03 compared with IAA-), and ICAs [greater than or equal to]10 JDF U combined with GAD antibodies gave a risk of 61% (P = 0.018). The risk for ICAs [greater than or equal to]10 JDF U with antibodies to 37-kDa antigen was 76% (P < 0.0001). Risk increased with the number of autoantibodies, from 8% for ICAs alone to 88% with [greater than or equal to]3 autoantibodies (14 cases detected) (P < 0.0001). The increased risk associated with multiple antibodies was observed independent of age. The median time to diagnosis in those with antibodies to 37- and/or 40-kDa antigen was 1.5 years, compared with 7.2 years in those with IAAs and GAD antibodies in the absence of antibodies to 37/40 kDa. The intensity and range of the autoantibody response offers better overall prediction of diabetes than any single autoantibody specificity, although antibodies to 37-/40-kDa antigens may prove to be useful markers of early clinical onset. We found that 78% of future cases of IDDM in [ICA.sup.+] relatives came from the 27% with multiple autoantibodies and estimate that 88% of individuals within this category will need insulin treatment within 10 years. We propose a simple predictive strategy based on these observations. Diabetes 43:1304-1310, 1994

Risk of progression to insulin-dependent diabetes mellitus (IDDM) can be assessed with reasonable accuracy in unaffected first-degree relatives of a child with the disease, and such estimates have been used to design clinical trials of agents that may delay or prevent the onset of diabetes (1). Islet cell antibodies (ICAs) offer highly sensitive prediction when measured by an assay with a low threshold of detection. We found that 82% of those who developed diabetes within 10 years had ICAs [greater than or equal to]10 Juvenile Diabetes Foundation units (JDF U) on entry into the Bart's-Windsor family study (2). ICAs do, however, offer less specific prediction, because only 40% of relatives with ICAs [greater than or equal to]10 JDF U would be expected to develop IDDM within 10 years (2)(3). In contrast, loss of the first-phase insulin response to intravenous glucose is a highly specific marker of progression, giving a 90% risk over 4 years (4), but it is relatively insensitive, identifying around 20% of those family members who will develop diabetes within 5 years (1). Other potential autoantibody markers include antibodies to insulin (IAAs)(5), to glutamic acid decarboxylase (GAD)(6), and to other islet proteins detectable as [M.sub.r] 37,000 and [M.sub.r] 40,000 proteolytic fragments (37- and 40-kDa antigens)(7). We set out to examine the predictive value of these additional humoral markers in a large population of [ICA.sup.+] relatives, with the aim of developing an approach to screening that would combine the sensitivity of ICA with improved specificity.

RESEARCH DESIGN AND METHODS

The Bart's-Windsor and Bart's-Oxford prospective family stuides have recruited parents and siblings of patients with IDDM diagnosed before age 21 from within the Oxford Regional Health Authority area in England. By 1 March 1992, 2,722 nondiabetic first-degree relatives had been screened, of whom 109 had been found to have ICAs [greater than or equal to]10 JDF U with detectable ICAs (<4 JDF U) on at least one other occasion. In this analysis, we have included 101 relatives (48 parents and 53 siblings) who fulfilled these criteria. Serum from the remaining eight was no longer available for study. The median age at entry to the study was 19.8 years (range 2-56 years). Subjects were followed for up to 14.3 years (median 3.8 years) with repeated sampling for ICAs and other autoantibodies. Eighteen (4 parents and 14 siblings) developed IDDM. The cumulative risk for the development of IDDM for the whole group and the duration of follow-up are summarized in Fig. 1.

[CHART OMITTED]

Autoantibody assays. Autoantibodies were all determined on the earliest available sample in which ICAs [greater than or equal to]10 JDF U were detected. Sequential

Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives.

samples taken at approximately annual intervals between study entry and diagnosis of IDDM were also tested where available. All assays were performed blind on coded samples.

ICAs. Undiluted sera were screened for conventional ICA-IgG by means of indirect immunofluorescence on 4-[micro]m cryostat sections of blood group O human pancreas (8). Positive samples were then titered by doubling dilutions in phosphate-buffered saline on tissue obtained from a single pancreas under standard incubation conditions (9). Local standard sera calibrated to 2, 4, 8, 16, 32, and 80 JDF U were included in each assay. End-point titers were converted to JDF U (10). The coefficients of variation (CVs) between assays for control sera with 8, 32, and 80 JDF U tested in 13 consecutive assays were 11, 7, and 6%, respectively, when expressed geometrically (SD [log.sub.2] JDF U/mean [log.sub.2] JDF U). The threshold of ICA detection was 4 JDF U.

IAAs. IAAs were assayed using a modification of the methods described by Palmer et al. (5) and Kurtz et al. (11). Sera were extracted using acid-washed, dextran-coated charcoal to remove endogenous insulin; 80 [micro]l of serum was then incubated for 48 h at 4[degrees]C with 80 [micro]l of 40 mmol/l phosphate buffer and 5.3 X [10.sup.-3] pmol radiolabeled human insulin (specific activity 2,000 Ci/mmol; Amersham, Amersham, U.K.), with and without excess (2.55 pmol/tube) cold insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark). The immunoglobulin fraction was precipitated using polyethylene glycol 6000 (12.9% wt/vol) and washed. The specific binding was calculated by subtracting the counts in the presence of cold insulin from the counts without the cold insulin. Results were expressed as percentage displaced binding. The CVs between assays for control sera with percentage displaced binding of 1, 10, and 50 were 25, 26, and 9%, respectively. Individuals were classified as [IAA.sup.+] if the corrected binding was > 3 SD above the mean of 172 adult blood donors (mean [+ or -] SD; -0.04 [+ or -] 0.26% displaced binding).

GAD antibodies. Antibodies to GAD in sera were measured by determining the enzyme activity immunoprecipitated by sera from a soluble extract of rat brain, as previously described (12). GAD activity immunoprecipitated was calculated relative to a standard positive serum included in each assay. The intra-assay CV was 15.4%. Sera were regarded as positive for anti-GAD antibodies if the relative antibody activity exceeded 2 SD of the activity in sera from a group of 30 healthy control individuals (mean [+ or -] SD; 6.2 [+ or -] 3.4% of positive control subjects). Using this assay, antibodies to GAD were found in 16 of 25 newly diagnosed patients with IDDM (12) and 1 of 28 control subjects (13).

Antibodies to 37-and 40-kDa islet antigens. Antibodies to 37-and 40-kDa islet antigens were measured by immunoprecipitation of [35.sup.S]methionine-labeled proteins from RIN 5AH cells extracted in Triton X-114 detergent as described previously (7). Immunoprecipitates were treated with trypsin (0.1 mg/ml) before sodium dodecyl sulfate-polyacrylamide electrophoresis and autoradiography. Serum samples were regarded as positive for antibodies to tryptic fragments of islet 64-kDa antigens if a band corresponding to the appropriate polypeptide could be detected on the autoradiogram. Antibody activities on positive samples were quantified by densitometric scanning of bands on autoradiograms expressing band density relative to that in a standard antibody-positive serum used in previous studies. Using this assay, antibodies to 37-and/or 40-kDa antigens were detected in 21 of 27 patients with newly diagnosed IDDM and in 0 of 26 control subjects (14).

ICA subtypes. ICAs have been subclassified on the basis of the ability of rat brain homogenate to inhibit islet staining in the ICA assay. This has been shown to correlate with staining pattern; those ICAs inhibited by rat brain homogenate give a [beta]-cell selective or restrictive pattern, while those not inhibited give a whole islet or unrestricted pattern (15). Inhibition experiments were performed on samples with ICAs [greater than or equal to] 20 JDF U using Wistar-Furth rat brain homogenate. Sera were preincubated overnight with either rat brain homogenate or homogenate buffer. Each was titered to end point in phosphate-buffered saline and tested in the ICA assay (15). Sera were classified as inhibited if the end-point titer in the sample preincubated with rat brain homogenate was two or more doubling dilutions less than that preincubated with homogenization buffer only. A control serum in which ICA staining was completely inhibited by rat brain homogenate and one in which staining was not inhibited were included in each assay.

Statistical analysis. Life tables were used to estimate the time to development of IDDM. Follow-up time for each subject was calculated from the date when ICAs [greater than or equal to] 10 JDF U were first detected. The start of insulin treatment was used as the date of diagnosis of IDDM. [x.sup.2] testing was used to assess associations between antibodies and with age. Survival experience was compared using the Lee-Desu statistic in SPSS-PC. Point estimates of risk are quoted as cumulative risk (95% confidence interval [CI]).

RESULTS

Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives.

Prevalence of autoantibodies. The combinations of antibodies detected in individuals grouped according to level of ICAs are shown in Fig. 2. Thirty-six individuals had ICAs alone, 38 had ICAs and one other antibody, 16 had two others, 7 had three others, and 4 had all the antibodies tested. ICAs [greater than or equal to] 80 JDF U were most frequent in children in the lowest age quartile, < 13.2 years of age (P = 0.03). Antibodies to 37-and/or 40-kDa antigens were more frequent in those with ICAs [greater than or equal to] 80 JDF U (P < 0.0001) and in children in the lowest age quartile (P = 0.04), but IAAs and GAD antibodies were not significantly associated with ICA titer or age. There were no significant associations between IAAs, antibodies to GAD, and antibodies to 37-and/or 40-kDa antigens. Multiple antibodies were detected more frequently in individuals with ICAs [greater than or equal to] 80 JDF U (P < 0.05).

[CHART OMITTED]

Family members who developed IDDM during followup. The characteristics of the 18 family members who developed IDDM and the autoantibodies detected are shown in Table 1. The median time to diagnosis of IDDM was 2.7 years.

[TABULAR DATA OMITTED]

Effect of age. Of the 25 individuals who were < 13.2 years of age at entry into the study, 10 developed IDDM, compared with 4 of 25 family members in the second age quartile (aged between 13.2 and 19.5 years), 3 of those in the third quartile (aged 19.5 to 40.7 years), and 1 in the highest age quartile. The cumulative risk of diabetes within 10 years was 62% (CI 33-92%) in the youngest age-group, 40% (CI 6-75%) in the second quartile, 35% (CI 0-74%) in the third quartile, and 4% (CI 0-22%) in those > 40.7 years of age (P = 0.02).

Quantitative measurement of ICAs. Of the 49 individuals with ICAs between 10 and 19 JDF U, 5 developed IDDM; the cumulative risk of IDDM within 10 years in this group was 34% (CI 8-60%). Of the 38 with ICAs of 20-79 JDF U, 7 became diabetic, giving a 10-year cumulative risk of 52% (CI 16-88%). Of the 14 with ICAs [greater than or equal to] 80 JDF U, 6 developed IDDM. The cumulative risk of IDDM within 5 years was 63% (CI 33-100%). Only one individual in this group remained nondiabetic after 5 years of follow-up. The survival curves were significantly different in the three groups (P < 0.0001) (Fig. 3A).

[CHART OMITTED]

ICA subtype. Inhibition of ICA staining with rat brain homogenate could be examined in 44 individuals with high titer ICAs ([greater than or equal to] 20 JDF U); this method could not be applied to those with lower titers. ICAs were inhibited in seven; one of these developed IDDM after 5 years. Rat brain homogenate did not inhibit ICA staining in 29, and in 8 samples, the results were equivocal (combined risk 61% after 10 years, CI 31-90%). The risk of diabetes was not significantly increased by the exclusion of individuals in whom ICA staining was not inhibited.

IAAs. IAAs were positive in 37 family members, of whom 11 developed IDDM after a median follow-up of 3.2 years. The cumulative risk after 10 years was 84% (CI 55-100%) in [IAA.sup.+] and 23% (CI 6-41%) in [IAA.sup.-] individuals, and the survival curves were significantly different (P = 0.03). Five family members developed diabetes between 4 and 8.5 years after entry, so that after only 5 years of follow-up, there was no significant difference in survival curves of [IAA.sup.+] and [IAA.sup.-] groups (cumulative risk at 5 years 25% [CI 7-42%] in [IAA.sup.+] vs. 12% [CI 2-22%] in [IAA.sup.-]) (Fig. 3B).

[CHART OMITTED]

GAD antibodies. Antibodies to GAD were detected in 40 family members overall and in 12 of those who developed diabetes. The difference in the survival curves for groups with and without GAD antibodies after 10 years was not statistically significant (P = 0.18) (Fig. 3C).

[CHART OMITTED]

Antibodies to 37-and 40-kDa antigens. Antibodies to the 37-and 40-kDa islet antigens were found in 13 individuals. An additional four had antibodies to the 40-kDa antigen only. Nine of the patients who developed IDDM had antibodies to one or both antigens. All of these developed diabetes within 5 years of study entry. The median time to diabetes was 1.5 years. The cumulative risk of IDDM within 5 years was 76% (CI 46-100%) in those with 37-kDa antibodies compared with 7% (CI 0-15%) in those without (P < 0.0001). After 10 years, a total of nine cases had been diagnosed in 37/40 kDa antibody-negative family members, and the risk in this group was 38% (CI 17-60%) (Fig. 3D). The cumulative risk of IDDM within 5 years in those with antibodies to 40-kDa antigen was 61% (CI 33-85%).

[CHART OMITTED]

Combining autoantibodies. The risk of diabetes increased with the number of antibody specificities detected. Of 36 individuals (36% of the cohort) with ICA alone, only 1

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developed IDDM. The cumulative risk of IDDM within 10 years in this group was 6% (CI 0-18%). Of the 38 individuals with ICA and one other autoantibody, 3 developed IDDM; the cumulative risk of IDDM by 10 years was 27% (CI 0-54%). The group of 27 family members who had at least three of the five autoantibodies tested contributed 14 of the 18 cases in the study (median time to diabetes 2.0 years, range 0.2-8.5 years), and the cumulative risk of IDDM within 10 years was 88% (CI 66-100%) (Fig. 4).

[CHART OMITTED]

This effect was also seen in family members with moderate levels of ICAs. Of the cohort, 87 had ICAs between 10 and 79 JDF U and 35 had ICAs alone; one of these developed IDDM, giving a cumulative risk of IDDM by 10 years of 6%. An additional 38 had this level of ICA and a single additional antibody; 3 of these developed IDDM, giving a cumulative risk of IDDM within 10 years of 30%. Of the 19 individuals with this level of ICA in association with two or more additional antibodies, 8 developed IDDM, and the cumulative risk of IDDM within 10 years was 84% (CI 56-100%).

The relationship between risk of diabetes and the number of antibodies appeared to be independent of age. The risk increased with the number of antibodies within each age quartile. In the youngest quartile, the cumulative risk of IDDM within 10 years associated with ICAs alone was 0%, with two antibodies was 12%, and with three or more antibodies was 88% (P = 0.003). In the second quartile, the risks were 15, 50, and 100% (NS); in the third quartile, risks were 0, 20, and 63% (NS), and in the oldest quartile, they were 0, 0, and 67%, respectively (P = 0.03).

Family members with three or more autoantibodies, who were 37-and/or 40-kDa antibody positive, had a median time to diagnosis of 1.5 years compared with 7.2 years in those with ICAs, IAAs, and GAD antibodies in the absence of 37-or 40-kDa antibodies. The cumulative risks of IDDM within 5 years were 64% (CI 37-91%) in the former group and 9% (CI 0-25%) in the latter. By 9 years, however, the cumulative risk of IDDM in those with ICAs, IAAs, and antibodies to GAD in the absence of 37-or 40-kDa antibodies was 100%, and the survival curves over the whole study period were not significantly different.

Changes in autoantibodies before diagnosis. Serial samples were examined from 14 of the family members who developed diabetes. Up to four samples per case were tested. Most individuals showed the same combination of antibody specificities throughout follow-up. The only changes in IAAs and antibodies to GAD were that one child (case 5) was [IAA.sup.-] at study entry but had high levels of the autoantibodies 4 months before diagnosis, and one parent (case 18) had GAD binding of 85% at study entry 8.1 years before diagnosis, but this was within the normal range in later samples. Sequential samples were tested from eight of nine individuals who were initially 37-and 40-kDa antibody negative and who later developed diabetes. These latest samples were taken between 0.3 and 2.7 years before insulin was started. Only one individual (case 10) appeared to develop 37- or 40-kDa antibodies between entry into the study and onset of diabetes. The antibodies were weakly positive for the first time in the last sample taken 2.7 years before diagnosis, having been negative in samples taken 30 and 18 months earlier.

DISCUSSION

All screening procedures aim for high sensitivity, to avoid missing future cases, combined with sufficient specificity to avoid false-positive results and unnecessary treatment. These aims inevitably conflict, because the specificity of a screening method is reciprocally related to its sensitivity. The more confident we become that individuals in a certain category will develop diabetes, the greater the proportion of those at risk we thereby exclude from the possible benefits of intervention. Highly sensitive prediction of IDDM in family members can be achieved with ICAs, provided an assay with a low detection threshold is used (2)(3). In the Gainesville study, 13 of 40 future cases had ICAs < 10 JDF U at entry into the study (3), while current analysis of the Bart's-Windsor and Bart's-Oxford family studies shows that 7 of 28 future cases were [ICA.sup.-] (< 4 JDF U) at entry, and that 3 of these had detectable ICAs during follow-up. We set out to develop an approach that would retain the sensitivity of ICAs as an initial screening procedure, while enhancing their predictive value by combined analysis with other diabetes-associated autoantibodies.

High-titer ICAs offer more specific prediction (2), but raising the ICA threshold from 10 to 20 JDF U only increased the cumulative risk from 43 to 53% after 10 years, while excluding 5 of 18 cases. Some ICAs are inhibited by rat brain homogenate, stain predominantly [beta]-cells ([beta]-cell-selective or restricted ICAs), and carry a lower risk of progression than ICAs that are not inhibited by rat brain homogenate (15)(16). These differences in ICA can currently only be assessed in sera with higher titers of ICA; only 44 could be evaluated, of which 7 were inhibited. Although this subtype was associated with a relatively low risk of IDDM, elimination from the analysis of those with ICAs [greater than or equal to] 20 JDF U only raised the cumulative risk from 53 to

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61% (not significant).

IAAs and antibodies to GAD and to 37- and 40-kDa islet antigens are all strongly associated with IDDM. IAAs and antibodies to 37- and 40-kDa antigens achieved a significant increase in cumulative risk in [ICA.sup.+] individuals, but in each patient, this was associated with loss of sensitivity. IAAs were the most useful marker in combination with ICAs, increasing the risk at 10 years to 84%, but seven cases were [IAA.sup.-] at initial testing. In contrast with previous studies (17), IAAs did not distinguish those with rapid onset of disease. Antibodies to GAD were present in 66% of those who progressed to diabetes but did not significantly increase the level of risk. Antibodies to 37-kDa antigen were, in contrast, highly specific markers for IDDM, associated with a 76% risk of IDDM within 5 years, but were found in only 50% of future cases. This confirms observations in monozygotic twins discordant for IDDM (12) and patients with polyendocrine autoimmunity (18).

Clear advantages emerged when all the markers were analyzed in combination. We found that 27 of the original cohort and 14 of 18 of those [ICA.sup.+] relatives who progressed to diabetes had two or more additional autoantibodies (Fig. 4). In other words, 78% of future cases came from 27% of the [ICA.sup.+] population. Life-table analysis shows that individuals in this category have an estimated 88% risk of developing diabetes within 10 years, irrespective of the presence or absence of metabolic abnormalities.

ICAs [greater than or equal to] 80 JDF U are associated with a very high risk of progression to IDDM, and excluding those with ICA staining inhibited by rat brain homogenate from the analysis increased the cumulative risk to 100% within 5 years. There is, therefore, little scope for improving prediction in this group. Only 6 of 18 individuals who progressed to IDDM fell into this category, however. Since 12 cases came from those with ICAs from 10 to 79 JDF U, conferring a cumulative risk of only 17% within 5 years and 40% within 10 years, this is the category within which we need to improve prediction. Our analysis suggests that this can be achieved by considering antibody markers in combination. Overall, 87 individuals had ICAs from 10 to 79 JDF U, and identification of those with at least two other autoantibody species enabled us to characterize a subgroup of 19 with an 84% risk of diabetes within 10 years. The approach is useful even with ICAs of 10-19 JDF U, because those with two other autoantibodies had a 63% risk of IDDM within 10 years. IAAs and antibodies to GAD proved particularly useful in those with ICAs between 10 and 79 JDF U. The detection of IAAs and/or antibodies to GAD identified over half the cases and were associated with risk at 10 years of 81 and 63%, respectively. Antibodies to 37- and 40-kDa antigens were associated with high titer ICAs and did not improve prediction in those with lower titers.

Because the risk of progression to diabetes is inversely related to age, and because children are more likely to have high levels of ICAs, antibodies to 37 kDa, and multiple antibodies, our findings might simply reflect these associations. This does not appear to be the case. A gradient of risk existed even within the youngest quartile, such that no children with ICAs alone developed IDDM, those with one other antibody had a cumulative risk within 10 years of 12%, and all those with two or more other antibodies developed diabetes. A similar pattern of risk is found at all ages. ICAs alone confer a low risk of IDDM in any age-group, while individuals with three or more antibodies had a cumulative risk of IDDM of 100% if aged < 20 years and of 66% if older than this. Analysis of the risk associated with different levels of ICAs, with IAAs, and with antibodies to GAD showed that the gradient of cumulative risk was similar within each age-group.

These findings imply that the intensity and range of the humoral autoimmune response determines overall risk. Our approach can therefore readily be extended to other candidates on the ever-growing list of anti-islet antibodies (19). Antibody type may, however, influence time to diabetes; antibodies to the 37- and 40-kDa antigens were detected when clinical onset of diabetes was imminent, while the combination of ICAs, IAAs, and antibodies to GAD was associated with a similar risk of diabetes over the 10 years, but was also found in those in whom clinical onset of diabetes was delayed. Antibodies to 37 or 40 kDa therefore appear to be associated with both high-titer ICAs and rapid progression to diabetes. Examination of sequential samples suggests that levels of antibodies to 37- and 40-kDa antigens change little during the disease prodrome, rather than appearing toward the time of diagnosis. This, in turn, suggests that they reflect true differences in the underlying disease process and not just a stage in its development.

An individual's probability of developing diabetes can be set out as a decision tree. Family history is the first major risk determinant, and the sibling of a child with IDDM within our region has an \3% risk of becoming diabetic within 10 years (1), rising to 43% in those with ICAs [greater than or equal to] 10 JDF U, and to 88% with the screening strategy we propose (Fig. 5). Even higher specificity can be achieved in those with loss of the first-phase insulin response in the intravenous glucose tolerance test (4), but metabolic screening of [ICA.sup.+] individuals will miss the majority of the at-risk population,

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at least from a single screening point (1). Further, those it identifies are likely to have advanced [beta]-cell damage, with a correspondingly reduced margin of benefit from therapy. We have estimated that ICAs are much less predictive of diabetes in those with no family history of the disease (20) and have proposed a screening strategy based on sequential analysis of genetic and immune markers for the general population. In family members, however, a logical approach would be to use ICA as the initial screen, using a low threshold assay, followed by testing for other antibodies in those who screen positive. Metabolic testing could then be used to its best effect within the high-risk category to identify those with and without end-stage prediabetes. The decision tree approach is sufficiently flexible to allow other characteristics, such as age (3), to be taken into account, and could allow precise targeting of those individuals who might benefit most from therapy.

[CHART OMITTED]

In conclusion, ICAs, despite technical limitations, remain sensitive markers of future IDDM. Prognosis is strongly related to the number of antibody specificities detected. The practical implication is that a single screening test promises to identify > 75% of future cases of IDDM in [ICA.sup.+] relatives, including those with relatively low levels of ICAs. The presence of antibodies to the 37- and 40-kDa antigens may supplement metabolic testing as an indication of the rate of progression to diabetes. This approach offers a simple potential means to identify candidates for intensive follow-up and for secondary prevention.

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REFERENCES

(1.)Bingley PJ, Bonifacio E, Gale EAM: Can we really predict IDDM? Diabetes 42:213-220, 1993

(2.)Bonifacio E, Bingley PJ, Dean BM, Shattock M, Dunger D, Gale EAM, Bottazzo GF: Quantification of islet-cell antibodies and prediction of insulin-dependent diabetes. Lancet 335:147-149, 1990

(3.)Riley WJ, Maclaren NK, Krischer J, Spillar RP, Silverstein J, Schatz DA, Schwartz S, Malone J, Shah S, Vadheim C, Rotter JI: A prospective study of the development of diabetes in relatives of patients with insulin-dependent diabetes. N Engl J Med 323:1167-1172, 1990

(4.)Vardi P, Crisa L, Jackson RA: Predictive value of intravenous glucose tolerance test insulin secretion less than or greater than the first percentile in islet cell antibody positive relatives of type 1 (insulin-dependent) diabetic patients. Diabetologia 34:93-102, 1991

(5.)Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, Paquette TL: Insulin antibodies in insulin-dependent diabetes before insulin treatment. Science 222:1337-1339, 1983

(6.)Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richler-Olesen H, de Camilli P: Identification of the 64K autoantigen in insulin-dependent diabetes as GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347:151-156, 1990

(7.)Christie MR, Hollands JA, Brown TJ, Michelsen BK, Delovitch TL: Detection of pancreatic islet 64,000 M, autoantigens in insulin-dependent diabetes distinct from glutamate decarboxylase. J Clin Invest 92:240-248, 1993

(8.)Bottazzo GF, Dean BM, Gorsuch AN, Cudworth AG: Complement fixing islet cell antibodies in type 1 diabetes: possible monitors of active [beta]-cell damage. Lancet 1:668-672, 1980

(9.)Bottazzo GF, Gleichman H: Immunology and Diabetes Workshops: report of the First International Workshop on Standardisation of Cytoplasmic Islet Cell Antibodies. Diabetologia 29:125-126, 1986

(10.)Bonifacio E, Lernmark A, Dawkins RL: Serum exchange and use of dilutions have improved precision of measurement of islet cell antibodies. J Immunol Methods 106:83-88, 1988

(11.)Kurtz AB, DiSilvio L, Bosi E: The determination of detection limits for insulin antibody assays. Diabetologia

Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives.

31:395-399, 1988

(12.)Christie MR, Brown TJ, Cassidy D: Binding of antibodies in sera from type 1 (insulin-dependent) diabetic patients to glutamate decarboxylase from rat tissues: evidence for antigenic and non-antigenic forms of the enzyme. Diabetologia 35:380-384, 1992

(13.)Christie MR, Tun RYM, Lo SSS, Cassidy D, Brown TJ, Hollands J, Shattock M, Bottazzo GF, Leslie RDG: Antibodies to GAD and tryptic fragments of the islet 64K antigen as distinct markers for development of IDDM: studies with identical twins. Diabetes 41:782-787, 1992

(14.)Christie MR, Vohra G, Champagne P, Daneman D, Delovitch TL: Distinct antibody specificities to a 64 kD islet cell antigen in type 1 diabetes as revealed by trypsin treatment. J Exp Med 172:789-795, 1990

(15.)Genovese S, Bonifacio E, McNally JM, Dean BM, Wagner R, Bosi E, Gale EAM, Bottazzo GF: Distinct cytoplasmic islet cell antibodies with different risks for type 1 (insulin-dependent) diabetes mellitus. Diabetologia 35:385-388, 1992

(16.)Gianani R, Pugliese A, Bonner-Weir S, Schiffrin A, Soeldner JS, Erlich HA, Awdeh Z, Alper CA, Jackson RA, Eisenbarth GS: Prognostically significant heterogeneity of cytoplasmic islet cell antibodies in relatives of patients with type 1 diabetes. Diabetes 41:347-353, 1992

(17.)Ziegler AG, Ziegler R, Vardi P, Jackson RA, Soeldner JS, Eisenbarth GS: Life-table analysis of progression to diabetes of anti-insulin autoantibody-positive relatives of individuals with type 1 diabetes. Diabetes 38:1320-1325, 1989

(18.)Christie MR, Genovese S, Cassidy D, Bosi E, Brown TJ, Lai M, Bonifacio E, Bottazzo GF: Antibodies to islet 37-kDa antigen but not to glutamate decarboxylase discriminate rapid progression to insulin-dependent diabetes mellitus in endocrine autoimmunity. Diabetes. In press

(19.)Harrison LC: Islet cell antigens in insulin-dependent diabetes: Pandora's box revisited. Immunol Today 13:348-352, 1992

(20.)Bingley PJ, Bonifacio E, Shattock M, Gillmor HA, Sawtell PA, Dunger DB, Scott RDM, Bottazzo GF, Gale EAM: Can islet cell antibodies predict IDDM in the general population? Diabetes Care 16:45-50, 1993

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Autoantibodies to Glutamic Acid Decarboxylase and Insulin in Islet Cell Antibody Positive Presymptomatic Type 1 Diabetes Mellitus: Frequency and Segregation by Age and Gender

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The frequency of antibodies to glutamic acid decarboxylase (GAD) and insulin (IAA) in presymptomatic Type 1 diabetes mellitus with a positive test for antibodies to islet cell antigen (ICA) was examined. Thirty-two persons positive for ICA (> 10 JDF units) were tested 2 to 48 months before their ascertained onset of Type 1 diabetes. ICA was quantitated by immunofluorescence as JDF units, anti-GAD by radioimmunoprecipitation and anti-insulin by radioimmunoassay. There was a positive test for anti-GAD in 25 (78 %), and for IAA in 23 (72 %), of the 32 prediabetic ICA-positive subjects. Stratification according to age at the onset of diabetes showed differing frequencies of anti-GAD and IAA in the prediabetic stage. Thus the positivity rate for anti-GAD for 18 subjects older than 10 years at onset of diabetes was 83 %, and for 14 aged 10 or younger at onset was 71 %; conversely, the rate for IAA for 18 subjects older than 10 at onset was 56 % and for 14 aged 10 or less at time of onset was 93 % ($p = 0.01$). The frequency of anti-GAD was higher in females (88 %) than males (71 %) whereas the frequency of IAA was higher in males (82 %) than in females (60 %). Since autoantibodies to GAD and insulin occur in presymptomatic Type 1 diabetes with differences in frequencies by age and gender, the stimuli to autoimmunity may operate differently at different ages, and may also be gender-related.

KEY WORDS **Type 1 (insulin-dependent) diabetes mellitus Islet cell autoantibodies Autoantibodies to glutamic acid decarboxylase Insulin autoantibodies**

Introduction

Type 1 (insulin-dependent) diabetes mellitus develops as a result of immunologically mediated destruction of pancreatic islet beta-cells in genetically susceptible individuals.[1] This is a chronic process in which there is a latent period of some years,[2] the presymptomatic or 'prediabetes' phase. Persons at risk of developing Type 1 diabetes can be recognized by the presence of autoimmune serological reactions[3,4] and decline in beta-cell insulin secretory capacity.[3]

First degree relatives of persons with Type 1 diabetes have an increased risk of developing diabetes.[4] Autoantibodies to islet cell cytoplasm (ICA),[5,6] insulin (IAA)[7] or the 64 kD antigen[8,9] now identified as glutamic acid decarboxylase (anti-GAD),[10] have been described in relatives in their prediabetic phase as long as 10 years before the onset of symptoms. These serological reactions could therefore predict the development of Type 1 diabetes. Also, individuals with prediabetes or recent onset Type 1 diabetes have augmented T lymphocyte responses to GAD,[11] suggesting that an autoimmune response to GAD is the initial determinant of Type 1 diabetes.[12]

ICA are known to be highly and specifically predictive of Type 1 diabetes in first degree relatives.[13] The predictive capacity of other autoantibodies has been less well studied although it has been suggested that anti-GAD may be equal to or superior to ICA in this respect.[4] In this paper, we compare the frequency of anti-GAD and IAA in stored sera from 32 Type 1 diabetic individuals selected for study according to a positive test for ICA in the prediabetic phase.

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Patients and Methods

Patients

The 32 study subjects, all of European descent, attended the Department of Paediatrics, School of Medicine, Auckland, New Zealand prior to the diagnosis of Type 1 diabetes. Their age at the clinical onset of Type 1 diabetes, sex, the method of ascertainment, and the period between the initial blood sample and the onset of overt diabetes are shown in Table 1. The subjects studied included all first degree relatives and some distant relatives of patients with Type 1 diabetes, and healthy children in a population-based school survey selected as previously described.[14] The 32 subjects (17 males, 15 females) who gave a positive test for ICA at a level of ≥ 10 JDF units included 23 (72 %) first degree relatives; all were kept under surveillance for a mean period of 14.2 months, range 2 to 48 months. The single blood sample tested for the present study was the latest available before the diagnosis of diabetes was established.

Methods

Islet cell antibody was determined by indirect immunofluorescence on cryostat sections of human pancreas.[15] The Auckland laboratory participates in the International ICA proficiency programme under the auspices of the Juvenile Diabetes Foundation (JDF); results from the laboratory have a validity, consistency, sensitivity, and specificity of > 95 % at all levels of positivity expressed

Table 1. Summary of findings for islet cell autoantibodies (ICA), insulin autoantibodies (IAA) and antibodies to GAD (anti-GAD) in the first serum sample obtained from the 32 Type 1 diabetic patients tested 2 to 48 months before onset of diabetes

Subject	Age at clinical onset (yr)	Sex	Ascertainment	Prediabetic period (months)	Autoantibodies		
					ICA (JDF units)	IAA[a] (units)	Anti-GAD[a] (units)
1	2	M	First degree relative	3	640	86.9	11
2	3	F	First degree relative	19	640	275	31
3	5	F	First degree relative	13	> 320	63.8	51
4	5	M	First degree relative	3	> 320	4.1	17
5	6	M	School study	4	320	12.6	12
6	7	F	School study	6	> 320	3.2	18000
7	8	M	First degree relative	9	> 320	152.1	14000
8	9	M	First degree relative	14	> 320	37.1	1000
9	9	F	School study	15	40	2.5	59
10	9	F	First degree relative	17	> 320	39.7	900
11	9	F	First degree relative	17	> 320	39.7	900
12	10	F	Family history[b]	16	> 320	17.5	14
13	10	F	First degree relative	2	20	3.0	53
14	10	F	School study	12	40	4.2	78
15	11	F	First degree relative	15	10	1.8	3750
16	11	M	First degree relative	11	320	4.2	54
17	11	M	Family history[b]	4	80	5.3	36
18	11	M	First degree relative	28	320	12.6	775
19	12	M	First degree relative	10	80	6.4	37
20	12	F	Family history[b]	18	1280	1.2	3750
21	12	M	School study	18	40	157	10
22	13	M	First degree relative	24	10	1.4	8
23	14	F	First degree relative	6	> 320	0.6	17
24	15	F	First degree relative	16	80	3.3	78
25	15	M	First degree relative	24	40	0.8	200
26	16	M	First degree relative	15	40	9.4	74
27	22	M	First degree relative	10	> 320	1.3	61
28	23	M	First degree relative	11	160	381.8	4375
29	31	F	Family history[b]	48	80	0.8	71
30	35	M	First degree relative	16	> 320	19.8	45000
31	35	M	First degree relative	16	> 320	19.8	15000
32	48	F	First degree relative	14	160	0.7	16000
Frequency (positive cases)					100%	71.9%	78.1%

[a]The upper limit of normal is defined as 2.9 units for IAA and 18 units for anti-GAD.
[b]Remote relative.

as JDF units, and findings relating to this have been reported previously.[15]

Antibody to glutamic acid decarboxylase was measured by a well-validated immunoprecipitation assay.[16,17] GAD was purified from porcine brain by affinity chromatography, iodinated with ^{125}I and preabsorbed with pooled normal human serum. The iodinated GAD contained 100 000 counts per min after preabsorption. Test sera in duplicate at a dilution of 1:2 were added and held overnight at 4 °C. Immune complexes were precipitated with protein A-Sepharose, and the radioactivity was measured. The level of anti-GAD in the samples was expressed as units of activity by comparing counts precipitated by test sera with counts precipitated by a reference serum defined to contain 100 U of activity, at a dilution of 1:2. Each assay run included the positive reference serum, known high positive, low positive and negative sera, and several sera from normal blood donors. The coefficients of variation for inter-assay and intra-assay replicates for a high positive serum (100 units) were 9 % (n = 13) and 7 % (n = 20), respectively, and for a low positive serum (30 units) were 14 % (n = 13) and 8 % (n = 20), respectively.[17] The levels of anti-GAD in 76 blood donors ranged from 4 to 18 units and were normally distributed with mean (±SD) of 9 (±3) units; thus 18 units (mean + 3 SD) was taken to define the upper normal limit.[17] With levels of anti-GAD in the range of 20–80 units in the immunoprecipitation assay there is a linear relationship between serum concentration and counts of labelled antigen precipitated, with a plateau at around 100 units. Accordingly, strongly reactive sera that gave values > 80 units in the immunoprecipitation assay were titrated in doubling dilutions and the end point was converted to assay units.[17] This assay was shown to have 100 % validity, sensitivity, and specificity in the 1st International Immunology and Diabetes Workshop on assays for anti-GAD.

IAA was measured by radioimmunoassay as described by Ziegler *et al.*[18] and standardized according to the procedure developed at the Joslin Clinic, Boston, Massachusetts, USA, by serum exchange through the courtesy of Dr G. Eisenbarth, Boston. In the assay procedure, essentially that reported by Vardi *et al.*,[19] 75 µl samples of serum were incubated with 25 µl 0.04 M phosphate buffer, pH 7.5, 0.05 % bovine serum albumin, and 0.025 % bovine gamma globulin. Another set of sera was incubated with 25 µl of the same phosphate buffer above, containing 4 µg ml^{-1} synthetic human insulin (Eli Lilly). 100 µl ^{125}I-porcine insulin was added to all tubes (10 000 cpm). This insulin was labelled locally and purified by polyacrylamide gel electrophoresis; the specific activity was 80 µl Ci µg^{-1}. Tubes were vortexed and spun to ensure all liquid was at the bottom of the tube, and held at 4 °C for 7 days. All tubes were counted for 1 min before precipitation with 1.5 ml cold 14.3 % polyethylane glycol (PEG) in Veronal buffer, pH 8.6, and 0.1 % Tween 20. The tubes were spun for 30 min at 4 °C, and the supernatant decanted and pellets washed twice with 11 % PEG in Veronal buffer. The pellets were drained for 10 min and counted for 10 min. The following calculation was used to derive a value for IAA:

$$\frac{\text{average cpm of pellets without cold insulin}}{\text{average total count}} - \frac{\text{average cpm of pellet with cold insulin}}{\text{average total count}}$$

Rather than adjusting values using the specific activity of tracer, as in Vardi *et al.*[19], we established an in-house standard by diluting an acquired insulin antibody to give a binding of 10 %. When the above calculation is multiplied by 1000, this in-house standard should give a value of 100 units; the values in each assay were adjusted up or down so that the standard always reads 100 units. We have adopted this approach to compensate for different batches of ^{125}I insulin and radio-label damage. The results are expressed as radioimmunoassay units, and levels of ≥ 3.0 units were considered positive, derived from 3SD above the mean (0.4 ± 0.9) for 105 normal subjects. In the two most recent Immunology and Diabetes Workshops, the assay performed with average 82 % sensitivity, 94 % specificity, 86 % validity, and 93 % consistency. The coefficient of variation at borderline values of 3.0 units is ± 22 %, but less at higher values.

Statistical Analysis

The statistical comparisons employed X^2 analysis with Yates correction.

Results

The levels of ICA, anti-GAD, and IAA for the 32 subjects who later developed Type 1 diabetes are shown in Table 1. As a criterion for inclusion, all patients were positive for ICA at levels from 10 to 1280 JDF units before diagnosis of diabetes. Anti-GAD was detected in 25/32 (78 %), up to 48 months before diagnosis, and levels ranged from 31 to 45 000 units. IAA was detected in 23/32 (72 %), and levels ranged from 3.0 to 382 units. In terms of concordance between autoantibodies before the diagnosis of diabetes, anti-GAD and IAA were present together in 18 (56 %) of the 28 subjects tested for both antibodies, anti-GAD was present singly in 7 (22 %), IAA were detected singly in 5 (16 %), and neither antibody was detectable in 2 (6 %). Thus either anti-GAD or IAA, or both, were present in 94 % of the subjects.

When the type of autoantibody present was examined according to the age of onset of Type 1 diabetes, there was a difference in frequency of anti-GAD and IAA, as shown in Table 2. Stratification of cases according to age at the time of eventual onset of symptomatic Type 1 diabetes showed that anti-GAD was non-significantly

Table 2. Frequency of autoantibodies to GAD (anti-GAD) and insulin (IAA) in prediabetic subjects according to age at eventual time of diagnosis of Type 1 diabetes. The figure shows data when stratification for onset of symptomatic Type 1 diabetes is at ≤ 10 or > 10, ≤ 12 or > 12 and ≤ 15 or > 15 years of age

Autoantigen positive	Age of onset (yr)					
	≤ 10 (*n* = 14)	> 10 (*n* = 18)	≤ 12 (*n* = 21)	> 12 (*n* = 11)	≤ 15 (*n* = 25)	> 15 (*n* = 7)
Anti-GAD	71.4 % (10)	83.3 % (15)	76.2 % (16)	81.8 % (9)	72 % (18)	100 % (7)
IAA	92.9 %[a] (13)	55.6 % (10)	76.2 % (16)	45.5 % (5)	76 % (19)	57.1 % (4)

[a] $\chi^2 = 5.42$ ($p = 0.02$) for IAA in ≤ 10 vs > 10 year group.
Figures in parentheses represent number of cases.

more prevalent in cases with an older age at onset than a younger age at onset, whereas IAA was significantly more prevalent in subjects with a younger age at onset. The data for a stratification at 10 years or less or older than 10 years show positivity rates for anti-GAD of 83 % (15 of 18 subjects) versus 71 % (10 of 14 subjects) whereas for IAA the corresponding rates were 56 % (10 of 18 subjects) versus 93 % (13 of 14 subjects) ($p = 0.01$ for IAA). The corresponding data for stratification at onset 12 years and 15 years are also shown in Table 2.

A gender difference was also noted for both anti-GAD and IAA. The frequency for positivity of anti-GAD was 71 % and 87 % for males and females, respectively, whereas the reverse applied for IAA, i.e. 82 % for males and 69 % for females. These findings were further examined in relation to the sex–age distribution of anti-GAD and IAA, either separately or in combination, in the sera of these prediabetic subjects. Those who were positive only for IAA were predominantly male (M:F ratio 4:1) and had a median age of onset of diabetes at 6 years, whereas those that were positive only for anti-GAD were predominantly female (M:F ratio 2:5) and had a median age of onset of 15 years. Subjects positive for both IAA and anti-GAD had an equal sex distribution and a median age of onset of 10 years (Table 3).

Discussion

'Preclinical diabetes' describes a state in which healthy individuals are presumed to have the histological lesion of Type 1 diabetes (insulitis) which will progress to overt IDDM over months or years. Seemingly no more than 200 individuals world-wide have been studied prospectively from non-diabetes to insulin dependency. In this study we examined the autoantibody profile of 32 such individuals identified initially by the presence of a positive test for ICA; of these, 25 (78 %) were positive for anti-GAD and 23 (72 %) for IAA. This frequency of anti-GAD in prediabetes is consistent with that of 74 % in 27 ICA-positive children with newly diagnosed Type 1 diabetes studied in Perth, Western Australia.[17] In that study, there were 11 patients positive for anti-GAD but negative for ICA, and presumably such anti-GAD positive and ICA-negative individuals would also occur in preclinical diabetes. However, the design of the present study precluded their identification. Our results, which confirm earlier observations on predictability of Baekkeskov *et al.*[8] and Atkinson *et al.*[9] pertaining to antibodies to the 64 kD antigen, were derived from a radioimmunoprecipitation assay using porcine brain GAD that is applicable to routine laboratory

Table 3. Frequency and case characteristics according to combinations of autoantibodies to insulin (IAA) and GAD (anti-GAD) in 32 subjects with prediabetes

	IAA+ *n* = 5 (16 %)	IAA+/anti-GAD+	anti-GAD+ *n* = 7 (22 %)	IAA-/anti GAD *n* = 2 (6 %)
Sex (M:F)	4:1	10:8	2:5	1:1
Age (yr)	6 (2–12)	10.5 (3–35)	15 (9–48)	13,14
Prediabetic (months)	4 (3–18)	13.5 (4–28)	15 (10–48)	10,6
IAA (units)	17.5 (4.1–157)	16.2 (3.0–382)	–	–
Anti-GAD (units)	–	427 (31–45 000)	200 (59–16 000)	–

Results as median (range).

use.[16] It provides a positivity rate of 69–80 % in newly diagnosed patients with rapid onset Type 1 diabetes,[16,17,20] similar to that in adults with a slow evolution to insulin dependency.[21–23] In the present study group, selected according to positivity for ICA and mainly first degree relatives of subjects with Type 1 diabetes and therefore at high risk,[6,13] a positive test for anti-GAD preceded the clinical onset of diabetes by at least 2 to 48 months.

Given that some 90 % of future cases of Type 1 diabetes will come from individuals with no family history of Type 1 diabetes, screening of populations for preclinical diabetes cannot be based only on relatives of known cases, nor on the technically demanding test for ICA. On the other hand, immunoassays for anti-GAD and IAA appear more promising.[4] However, it would be useful to know whether there is a sequential recruitment of autoimmune reactions to the various islet cell antigens in human Type 1 diabetes, as claimed for the NOD mouse model in which GAD is a prime candidate autoantigen for the induction of disease as well as a tolerogen that can prevent its occurrence.[24,25] At present, there are no sequential studies to indicate whether any one of the diabetes-associated autoantibodies regularly appears first. Positive levels of anti-GAD in the present cases covered a wide range of titration units as high as 45000. This does not seem to be in accord with the postulated protective effect of high levels of anti-GAD in prediabetic individuals as suggested by other workers.[11,26]

Our present study based on the preclinical stage of diabetes revealed an inverse frequency of anti-GAD and IAA according to the age of onset of diabetes and gender. Thus the frequency of anti-GAD was 78 % for the entire group, but was higher in subjects who were post-adolescent than pre-adolescent at the time of onset of diabetes. The converse applied for IAA in that the frequency was 72 % for the entire group, but was much higher in those with an onset of diabetes in pre-adolescence. This is in accord with data of Atkinson *et al.*,[9] since 87 % (20/23) of their patients with Type 1 diabetes with an onset at 10 years or after were positive for anti-GAD compared with only 60 % (3/5) in those with a younger age at onset. While there is a known higher frequency of IAA in patients who develop Type 1 diabetes before puberty,[19,27] the higher frequency of anti-GAD in post-adolescent than pre-adolescent cases of Type 1 diabetes has not been reported. Moreover, there was an excess of IAA in males, and an excess of anti-GAD in females as previously reported.[17]

As judged by data from humans and NOD mice, an autoimmune reaction to GAD with a T lymphocyte dependency may well be a primary cause of Type 1 diabetes,[24,25] and the present data and other studies from our laboratory on the presence of anti-GAD in the prediabetic phase of Type 1 diabetes are supportive of this. The alternative proposition, that the autoimmune response to GAD is secondary to destruction of the beta islet cells of the pancreas from other causes, being consequential rather than causal and thus irrelevant to pathogenesis, is still invoked in relation to anti-GAD and various other autoimmune responses. However, there are few sustainable examples of persisting autoimmune responses accompanying ongoing tissue injury due to identifiable extrinsic agents, although an initiating effect of extrinsic injury can seldom be excluded.

Some 20–25 % patients with Type 1 diabetes, whether preclinical or overt, remain negative for anti-GAD. This may be because a T-cell response to GAD predominates,[11] or because the autoimmune response is directed to islet cell autoantigens other than GAD. Notably entirely seronegative cases of Type 1 diabetes are quite infrequent, at least among Caucasians; in particular newly diagnosed diabetic children in Sydney, Australia showed an autoimmune response to either GAD, islet cell cytoplasm or insulin, singly or in combination, in all but 6 % of 173 cases studied.[20] Our present finding of different frequencies of anti-GAD and IAA in relation to age and gender suggests that different autoantigens, GAD, insulin or others, may be dominant according to circumstances. The reported differences in HLA type and autoantibody profiles in early onset and late onset Type 1 diabetes[28–30] could reflect HLA-dependent differences in antigen presentation to T cells at different ages from infancy to adult life. Comparative assessment of all three tests, ICA, IAA, and anti-GAD, including the relative predictive value for subsequent Type 1 diabetes for different age groups is now in progress in our laboratory.

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References

1. Eisenbarth GS. Type I diabetes mellitus. A chronic autoimmune disease. *N Engl J Med* 1986; 1360–1368.
2. Gorsuch AN, Spencer KM, Lister J, McNally JM, Dean BM, Bottazzo GF, *et al.* Evidence for a long prediabetic period in type I (insulin-dependent) diabetes mellitus. *Lancet* 1981; **11**: 1363–1365.
3. Srikanta S, Ganda OP, Soeldner JS, Eisenbarth GS. First degree relatives of patients with type I diabetes: islet cell anti-bodies and abnormal insulin secretion. *N Engl J Med* 1985; **313**: 461–464.
4. Palmer JP, McCulloch DK. Prediction and prevention of Type 1 diabetes—1991. *Diabetes* 1991; **40**: 943–9.
5. Bottazzo GF, Dean BM, Gorsuch AN, Cudworth AG, Doniach D. Complement fixing islet-cell antibodi

type I diabetes; possible monitors of active beta cell damage. *Lancet* 1980; **i**: 668–672.
6. Tarn AC, Thomas JM, Dean BM, Ingram D, Schwarz G, Bottazzo GF. Predicting insulin-dependent diabetes. *Lancet* 1988; **i**: 845–850.
7. Palmer JP, Asplin CM, Clemons R, Lyon K, Iaipati O, Raghu P, *et al.* Insulin antibodies in insulin dependent diabetes before insulin treatment. *Science* 1983; **222**: 1337–1339.
8. Baekkeskov S, Kristensen JK, Srikanta S, Bruining GJ, Mandrup-Poulsen T, DeBeaufort C, *et al.* Antibodies to a M 64000 human islet cell antigen precede the clinical onset of insulin dependent diabetes. *J Clin Invest* 1987; **79**: 926–934.
9. Atkinson MA, Maclaren NK, Scharp DW, Lacy PE, Riley WI. 64,000 M autoantibodies as predictors of insulin-dependent diabetes. *Lancet* 1990; **335**: 1357–1360.
0. Baekkeskov S, Aanstoot H-J, Christgau S, Reetz A, Solimena M, Cascalho M, *et al.* Identification of the 64K autoantigen in insulin-diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature (Lond)* 1990; **347**: 151–156.
1. Honeyman MC, Cram DS, Harrison LC. Glutamic acid decarboxylase 67–reactive T cells: a marker of insulin-dependent diabetes. *J Exp Med*; 1993: **177**: 535–540.
2. Solimena M, DeCamilli P. Diabetes—spotlight on a neuronal enzyme. *Nature* 1993; **366**: 15–17.
3. Bingley PJ, Bonifacio E, Shattock M, Gillmor HA, Sawtell PA, Dunger DB, *et al.* Can islet cell antibodies predict IDDM in the general population? *Diabetes Care* 1993; **16**: 45–50.
4. Elliott RB, Pilcher CC, Stewart A, Fergusson D, McGregor MA. The use of nicotinamide in the prevention of type 1 diabetes. In: Allison AC, Lafferty KJ, Pliri H, eds. *Immunosuppressive and Antiinflammatory Drugs*. New York: Annals of New York Academy of Sciences, 1993; 333–341.
5. Pilcher CC, Elliott RB. A sensitive and reproducible method for the assay of human islet cell antibodies. *J Immunol* 1990; **129**: 111–117.
6. Rowley MJ, Mackay IR, Chen Q-Y, Knowles WJ, Zimmet P. Antibodies to glutamic acid decarboxylase discriminate major types of diabetes mellitus. *Diabetes* 1992; **41**: 548–551.
7. Chen Q-Y, Byrne GC, Jones T, Tuomi T, Mackay IR, Rowley MJ, *et al.* Antibodies to glutamic acid decarboxylase in Australian children with insulin dependent diabetes mellitus and their first degree relatives. *Pediatric Res* 1993; **34**: 785–790.
8. Ziegler AG, Ziegler R, Vardi P, Jackson RA, Soeldner JS, Eisenbarth GS. Life-table analysis of progression to diabetes of anti-insulin autoantibody-positive relatives of individuals with Type 1 diabetes. *Diabetes* 1989; **38**: 1320–1325.
19. Vardi P, Dib SA, Tuttleman M, Connelly JE, Grinbergs M, Radizabeh A, *et al.* Competitive insulin autoantibody assay. Prospective evaluation of subjects at high risk for development of Type 1 diabetes mellitus. *Diabetes* 1987; **36**: 1286–1291.
20. Howard NJ, Verge CF, Tuomi T, Egan M, Arundel T, Hulinska H, *et al.* Autoantibodies at diagnosis in childhood IDDM: Contrast between GAD autoantibodies and other antibodies. *Diabetologia* 1993; **36**: (suppl1): A90.
21. Tuomi T, Groop LC,Zimmet PZ, Rowley MJ, Knowles W, Mackay IR. Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin dependent onset of disease. *Diabetes* 1993; **42**: 359–362.
22. Zimmet PZ, Tuomi T, Mackay IR, Rowley MJ, Knowles W, Cohen M, *et al.* Latent autoimmune diabetes mellitus in adults (LADA)—the role of antibodies to glutamic acid decarboxylase in prediction and diagnosis. *Diabetic Med* 1994: (in press).
23. Hagopian WA, Karlsen AE, Gottsäter A, Landin-Olsson M, Grubin CE, Sundkvist G, *et al.* Quantitative assay using recombinant human islet glutamic acid decarboxylase (GAD65) shows that 64K autoantibody positivity at onset predicts diabetes type. *J Clin Invest* 1993; **91**: 368–374.
24. Kaufman DL, Clare-Salzler M, Tian J, Forsthuber T, Ting GSP, Robinson P, *et al.* Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 1993; **336**: 69–72.
25. Tisch R, Yang X-D, Singer SM, Liblau RS, Fugger L, McDevitt HO. Immune response to glutamic acid decarboyxlase correlates with insulitis in non-obese diabetic mice. *Nature* 1993; **366**: 72–75.
26. Thai AC, Eisenbarth GS. Natural history of IDDM. *Diabetes Rev* 1993; 1: 1–14.
27. Arslanian SA, Becker DJ, Rabin B, Atchinson R, Eberhardt M, Cavender D, *et al.* Correlates of insulin antibodies in newly diagnosed children with insulin-dependent diabetes before insulin therapy. *Diabetes* 1985; **34**: 926–930.
28. Caillat-Zucman S, Garchon H-J, Timsit J, Assan R, Boitard C, Djilali-Saiah I, *et al.* Age-dependent HLA genetic heterogeneity of type I insulin dependent diabetes mellitus. *J Clin Invest* 1992; **90**: 2242–2250.
29. Serjeantson SW, Court J, Mackay IR, Matheson B, Rowley MJ, Tuomi T, *et al.* HLA DQ genotypes are associated with autoimmunity to glutamic acid decarboxylase in insulin-dependent diabetes mellitus patients. *Hum Immunol* 1993; **38**: 97–104.
30. Vandewalle CL, Decraene T, Schuit FC, DeLeeuw IH, Pipeleers DG, Gorus FK and the Belgian Diabetic Registry. Insulin autoantibodies and high titre islet cell antibodies are preferentially associated with HLA DQA1*0301-DQB1*-0302 haplotype at clinical onset of type 1 (insulin-dependent) diabetes mellitus before age 10 years, but not at onset between age 10 and 40 years. *Diabetologia* 1993; **36**: 1155–1162.

Antibodies to islet 37k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity.

by Michael R. Christie, Stefano Genovese, David Cassidy, Emanuele Bosi, Thomas J. Brown, Manuelita Lai, Ezio Bonifacio and Gian Franco Bottazzo

Apart from islet cell antibodies (ICAs), antibodies to glutamate decarboxylase (GAD), insulin autoantibodies (IAAs), and a novel islet antigen (37k antigen) are potential markers for insulin-dependent diabetes mellitus (IDDM). GAD is also an antigen in stiff-man syndrome (SMS), and both SMS and IDDM are associated with ICAs and autoimmunity to other endocrine organs. We investigated possible links between antibody responses to islet antigens with autoimmunity to other endocrine organs and determined which specific antibodies can identify individuals who progress to IDDM. Antibodies to GAD were detected in [great than or equal to]90% of both diabetic and nondiabetic patients with ICAs and other endocrine autoimmunity, in 59% of ICA-positive IDDM patients without endocrine autoimmunity, in all patients with SMS, but in only 1-3% of healthy (nondiabetic) and autoimmune disease control subjects. GAD antibody levels were increased in ICA-positive IDDM patients with polyendocrine autoimmunity compared with those without. In contrast, antibodies to 37k antigen were only detected in patients who developed acute-onset IDDM. IAAs were also associated with IDDM. Thus, certain factors enhance antibody responses to GAD in polyendocrine autoimmunity, but this does not necessarily lead to development of IDDM or SMS. Antibodies to 37k antigen are strongly associated with acuteonset IDDM and are useful serological markers for disease. Diabetes 43:1254-1259, 1994

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease first found to be associated with islet cell antibodies (ICAs) (1). More recently, antibodies to glutamic acid decarboxylase (GAD) have been detected in the majority of IDDM patients at and before onset (2-7), and T-cell reactivity against GAD has been reported in the peripheral blood of IDDM patients (8). However, GAD autoantibodies are not restricted to IDDM but are also detected in patients with stiff-man syndrome (SMS), a rare neurological disorder affecting the GABA-ergic ([gamma]-amino-n-butyric acid) nervous system (8,10). GAD autoimmunity in SMS is strongly associated with the presence of other endocrine/organ-specific autoimmune manifestations (10). Interestingly, we have also detected GAD antibodies in nondiabetic [ICA.sup.+] patients with autoimmunity to other endocrine organs (11). Common features of SMS and polyendocrine autoimmunity may point to possible links between autoimmune responses to GAD and to other endocrine organs. In both of these [ICA.sup.+] populations, which are predominantly female, IDDM develops in only \30% of individuals, despite very high titers of ICAs (10,12).

IDDM is associated with autoimmunity to islet antigens other than GAD, including insulin (13), and a 64,000 [M.sub.r] antigen that is distinct from GAD (14,15). Antibodies to the latter antigen (37k antigen) can be detected by measuring antibody binding to 37,000 and 40,000 [M.sub.r] fragments generated by tryptic proteolysis of the 64,000 [M.sub.r] antigen (14). Antibodies to the 37k antigen are more closely associated with progression to diabetes than are antibodies to GAD in identical twins of diabetic patients (16). In this study, we have investigated antibody responses to a variety of islet antigens in a cohort of patients with ICAs and other endocrine autoimmunity and have determined which of these antibodies identify individuals who progress to IDDM.

RESEARCH DESIGN AND METHODS

[ICA.sup.+] endocrine autoimmune patients. Endocrine autoimmune patients positive for ICA were selected from a large prospective study on the pathogenesis of IDDM (U.K. Polyendocrine Study) (12). The basis for recruitment was the detection of ICA on at least two occasions in the absence of IDDM and the presence of at least one other organ-specific antibody and/or organ-specific autoimmune disease. The project was initiated in 1985, and 186 individuals are currently enrolled. Participants have been followed prospectively for the development of IDDM. Two groups of patients were selected for this study. The first group included all patients in the U.K. Polyendocrine Study who progressed to IDDM and who had a serum sample available before, or <1 year after, disease diagnosis. Seventeen patients (11 women) met these criteria, and the earliest serum sample and a serum sample drawn close to diabetes onset were selected for antibody analysis. Of these 17 patients, 9 required insulin at diagnosis of diabetes, and 1 additional patient required insulin treatment within.4 months of diagnosis of non-insulin-dependent diabetes mellitus (NIDDM) (acute-onset diabetes). The mean age ([+ or -] SE) at onset of IDDM for these patients was 51.0 [+ or -] 6.1 years (range 17-75). The remaining 7 patients (4 women) had NIDDM at initial presentation and did not require insulin for >1 year (range 15-48 months) before progression to IDDM (slow-onset diabetes). In these patients, the mean age at diagnosis of IDDM was 54.3 [+ or -] 8.3 years (range 13-79). The second group consisted

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of 20 patients (15 women) selected at random from participants in the U.K. Polyendocrine Study who had ICAs > 10 Juvenile Diabetes Foundation units (JDF U) and who had not developed diabetes on follow-up for at least 36 months (mean 67.7 months, range 36-102). [ICA.sup.+] IDDM patients. A group of 22 patients (9 women) with acuteonset IDDM were included in the study. The mean age of the patients at serum sample was 16.4 [+ or -] 1.9 years (range 5-40). Serum samples were obtained within 12 months of diagnosis of disease. These patients were selected for having ICAs but no detectable antibodies to thyroglobulin, thyroid microsomes, or gastric parietal cells.

SMS patients. Six patients (four women) with SMS were included in the study. The mean age at serum sampling was 53.8 [+ or -] 2.5 years (range 47-63). Three SMS patients also had IDDM. All SMS patients had high titer ICAs (>80 JDF U).

Control groups. The first group included 111 [ICA.sup.-] nondiabetic patients who had other autoimmune diseases. Of these, 30 patients had autoimmune thyroid disease, 10 had Addison's disease, 10 had pernicious anemia, 10 had autoimmune polyendocrine disease, 27 had rheumatoid arthritis, and 24 had systemic lupus erythematosus (SLE). The second control group comprised 80 healthy individuals with no known family history of IDDM or other autoimmune diseases.

ICAs. These were measured by indirect immunofluorescence (IFL) on 4-mm cryostat sections of blood group O human pancreas, as described previously (17). Positive samples were titrated to end point in doubling silutions in 10 mmol/l phosphate-buffered saline (pH 7.2). Local standard sera calibrated to 2, 4, 8, 16, 32, and 80 JDF U were included in each assay. End-point titers of test samples were converted to JDF U by comparison with a standard curve of [log.sub.2] JDF U versus [log.sub.2] of end-point titer of the standard sera. The threshold of ICA detection was 5 JDF U. Insulin autoantibodies (IAAs). IAAs were measured by radioimmunoassay, with displacement with unlabeled insulin to correct for nonspecific binding (18). The mean ([+ or -] SD) corrected binding for 140 control sera was 0.149 [+ or -] 0.298%. Sera with binding >3 SD above the mean for these control sera were considered positive. Since the introduction of the Immunology and Diabetes Workshops for the standardization of ICA and IAA, our laboratories have repeatedly participated with a high degree of performance.

Antibodies to GAD. These were measured by determining the enzyme activity immunoprecipitated by antibodies in sera from a soluble extract of rat brain as described previously (3). Serial dilutions of selected sera from each group demonstrated an approximately linear relationship between antibody concentration and enzyme activity immunoprecipitated for most samples. However, dilution experiments with all SMS patients demonstrated a marked prozone effect at low dilutions of serum. For quantification of GAD antibody levels, sera from SMS patients were analyzed at a dilution of 1:250 and all other sera at 1:5. Enzyme activity immunoprecipitated was calculated relative to that of the same standard antibody-positive control serum used in previous analyzes of GAD antibody activities (14,16). Sera were regarded as positive if the relative antibody activity exceeded 2 SD of the activity in sera from a group of 30 healthy (nondiabetic) control individuals (mean [+ or -] SD; 6.2 [+ or -] 3.4% of positive control). The interassay coefficient of variation was 15.4%.

Antibodies to 50,000, 40,000, and 37,000 [M.sub.r] fragments of islet 64,000 [M.sub.r] antigens. These antibodies were measured by immunoprecipitation of [[35.sup.S]]methionine-labeled polypeptides released from particulate fractions of neonatal rat islets by trypsin treatment as described previously (14). Serum samples were regarded as positive for a specific antibody activity if a band corresponding to the appropriate polypeptide could be detected on the autoradiogram. Antibody levels were quantified by densitometric scanning of bands on autoradiograms, expressing band density as a proportion of a standard antibody-positive control serum included in each antibody analysis (14).

Other autoantibodies. Antibodies to thyroglobulin (TG-Ab) and thyroid microsomes (TM-Ab) were detected by indirect hemagglutination using a commercial kit (Serodia, Japan). The threshold titer for antibody positivity was 1/20 for TG-Ab and 1/400 for TM-Ab. Antibodies to gastric parietal cells (GPC-Ab) were detected by IFL using 4-mm cryostat sections of human blood group O stomach (19).

Statistical analysis. The significance of differences between antibody frequencies in populations was determined by [X.sup.2] analysis with Yates' correction or by Fisher's exact test as appropriate. The significance of differences between antibody levels in patient groups was determined by the Mann-Whitney U test. The degree of correlation between antibody levels and titers was calculated as the Spearman coefficient of rank correlation.

RESULTS

Antibodies to brain GAD and 50,000 [M.sub.r] fragments of islet GAD. Antibodies to GAD were measured by two independent assays: 1) by measuring GAD enzyme activity immunoprecipitated from extracts of rat brain (GAD

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antibodies) and 2) by immunoprecipitation of 50,000 [M.sub.r] fragments of rat islet antigen, previously shown to be islet GAD (15) (50k antibodies). GAD and 50k antibodies were detected in 18 (90%) and 19 (95%), respectively, of 20 [ICA.sup.+] endocrine autoimmune patients without IDDM, in all 17 [ICA.sup.+] endocrine autoimmune patients who developed IDDM, and in all 6 patients with SMS (Table 1). The two [ICA.sup.+] endocrine autoimmune patients who were negative for GAD antibodies had low titer ICA (Table 2). When present, GAD antibodies persisted with time; no patients converted from antibody negative to positive, and the antibodies were detected 84 months before diabetes onset in one patient (patient 4 in Table 2) and persisted for at least 6 years in [ICA.sup.+] endocrine autoimmune patients who did not develop diabetes. GAD antibodies in the absence of antibodies to 37k antigen or IAAs were found in 23 [ICA.sup.+] polyendocrine disease patients; 17 of these are nondiabetic, 5 progressed to IDDM slowly after a period of NIDDM, and 1 developed acute-onset IDDM (Table 2). Of the 22 [ICA.sup.+] IDDM patients without endocrine autoimmunity, 13 (59%) had GAD antibodies and 13 (59%) had 50k antibodies. Five of nine GAD antibody-negative IDDM patients had high titer ICAs (>80 JDF U), and GAD antibodies were not correlated with ICA titers in this group.

A quantitative analysis of GAD enzyme activity immunoprecipitated by antibodies in sera of [ICA.sup.+] patients is shown in Fig. 1. No significant difference was observed between GAD antibody levels in diabetic and nondiabetic [ICA.sup.+] endocrine autoimmune patients. However, GAD antibody levels in [ICA.sup.+] IDDM patients without endocrine autoimmunity were significantly lower than those in both diabetic and nondiabetic [ICA.sup.+] endocrine autoimmune groups (P [less than or equal to] 0.002). SMS patients all had high GAD antibody levels (Fig. 1).

[CHART OMITTED]

GAD antibodies were rarely detected in the [ICA.sup.-] groups. None of the 51 patients with rheumatoid arthritis or SLE were positive for GAD antibodies, and only 2 patients with endocrine autoimmune disease were positive, 1 with Graves' disease and 1 with Addison's disease and hypothyroidism (Table 3). The latter patient was also positive for 50k antibodies. Two of 80 (2.5%) normal control individuals were positive for antibodies to brain GAD and 50,000 [M.sub.r] tryptic fragments of the islet antigen.

[TABULAR DATA OMITTED]

Antibodies to islet 37k antigen. In contrast to antibodies to GAD, antibodies to 37k antigen were only found in individuals who developed IDDM (Tables 1 and 2). Overall, 9 of 17 (53%) [ICA.sup.+] endocrine autoimmune patients who progressed to IDDM were positive for antibodies to 37k antigen. Nine of 10 patients (90%) with acute-onset IDDM had these antibodies, whereas all patients with slow-onset IDDM were negative ($P < 0.005$). Two patients with acute-onset IDDM converted from antibody negative to positive on follow-up, in one patient (patient 4) between 84 and 26 months before diabetes onset and in a second (patient 9) between 7 and 2 months before diagnosis. Of the six patients with SMS, two had antibodies to 37k antigen and both of them had IDDM. Antibodies to 37k antigen were detected in 18 of 22 (82%) [ICA.sup.+] IDDM patients without endocrine autoimmunity. ICA titers were significantly correlated with levels of antibodies to 37k antigen in these patients ($r = 0.71$, $P < 0.001$). Antibodies to 37k antigen were not detected in any of the 111 [ICA.sup.-] patients with autoimmune disease (Table 3) or in the 80 healthy control individuals.

Antibodies to insulin. IAAs were measured only in [ICA.sup.+] endocrine autoimmune patients (Tables 1 and 2); in patients who developed IDDM, IAAs were measured in samples collected before initiation of insulin treatment. IAAs were detected in 4 of 10 (40%) patients who developed acute-onset IDDM, in 2 of 7 (29%) slow-onset IDDM patients, and in 1 of 20 (5%) nondiabetic individuals. The frequency of IAAs in acute-onset IDDM patients was significantly higher than that in [ICA.sup.+] endocrine autoimmune patients who have not developed IDDM ($P < 0.05$).

Thyrogastric antibodies. [ICA.sup.+] endocrine autoimmune patients and SMS patients had high frequencies of TG-Ab, TM-Ab, and GPC-Ab (Tables 1 and 2). The frequency of thyrogastric antibodies in [ICA.sup.-] patients was similar to that expected for each group of diseases tested (Table 3).

DISCUSSION

A major goal of research into IDDM is to define genetic and/or immunological markers that precisely identify individuals predisposed to the disease (20). GAD is one of a number of novel islet cell antigens known to be associated with IDDM. However, GAD autoimmunity also develops in the absence of diabetes. Thus, GAD is an autoantigen in autoimmune SMS, but only one-third of SMS patients develop IDDM. We have also detected GAD antibodies in \20% of nondiabetic identical twins who are long-term discordant for diabetes with their diabetic co-twin (16) and who are therefore at low risk for diabetes (21).

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Furthermore, GAD has been shown to be a target for a [beta]-cell-selective ICA that is not predictive of diabetes development, in contrast with whole-islet ICA, which is associated with disease (11,22).

To further characterize the associations of GAD autoimmunity with diabetes and other autoimmune diseases, we have analyzed GAD antibodies in [ICA.sup.+] and [ICA.sup.-] patients with endocrine autoimmunity. GAD antibodies, including antibodies to 50,000 [M.sub.r] fragments of islet [GAD.sub.65] that correlate well with the former (3), were detected in almost all [ICA.sup.+] endocrine autoimmune patients, whether or not they developed diabetes. GAD antibody levels were detected at similarly high levels in both diabetic and nondiabetic groups. The elevated frequency of GAD antibodies in this cohort of endocrine autoimmune patients, together with the strong association of GAD autoimmunity in SMS with the presence of ICA and other organ-specific autoimmunities (10), suggested possible links between endocrine autoimmunity and immune responses to GAD. However, GAD antibodies appeared in <2% of patients with autoimmune disease who were negative for ICA. Interestingly, when detected, the antibodies appeared only in endocrine autoimmune patients and not in patients with non-organ-specific disease. GAD antibodies thus appear predominantly in the [ICA.sup.+] endocrine autoimmune population. It has been estimated that 2.4% of patients with autoimmune disease possess ICA (23) (and therefore most likely GAD antibodies), a proportion similar to the frequency of GAD antibodies in our healthy control subjects (2.5%). Thus, development of GAD antibodies is not strongly associated with endocrine autoimmunity per se, other than autoimmunity to pancreatic islet cells or GAD-rich cells in the brain.

Despite this lack of association, the levels of GAD antibodies in IDDM patients with endocrine autoimmunity were significantly higher than in those who lacked organ-specific antibodies. These results suggest that, although the appearance of antibodies to GAD may not be dependent on autoimmune responses to other organs, the intensity of the antibody response to the enzyme, when it does develop, is enhanced in patients with coexistent endocrine autoimmunity. This concept is supported by a previous study, in which high levels of GAD antibodies were predominantly detected in diabetic patients with other organ-specific antibodies (24). Our series concentrated mainly on adult patients with type II autoimmune polyglandular syndrome (Schmidt syndrome extended [25]), but similar high-titer GAD antibodies have also been described in type I autoimmune polyglandular syndrome, which primarily affects children (26).

Although possessing high levels of autoantibodies to GAD, only 30% of the [ICA.sup.+] endocrine autoimmune patients progress to IDDM (12). In another study (27), we had the opportunity to examine the pancreases of three such patients who died without developing IDDM. These pancreases displayed none of the immunohistological changes that are normally associated with autoimmune destruction of pancreatic [beta]-cells in IDDM, suggesting that the strong antibody responses to GAD in these individuals occur independently of lymphocytic infiltration of islets, a potent indicator of [beta]-cell destruction.

This study identifies a unique nondiabetic population, initially defined as possessing endocrine autoimmunity with ICA, with a high frequency ([greater than or equal to]90%) and high levels of GAD antibodies, but which, in most cases, lacks other serological markers of IDDM. Is it possible that GAD antibodies, when they develop in the absence of other diabetes-associated immune markers, play a neutral, if not protective, role in determining susceptibility to IDDM? To try to answer this question, we investigated whether antibodies to insulin and 37k antigen could distinguish those GAD antibody-positive individuals who progressed to IDDM. Both markers were indeed associated with disease. IAAs were detected in 6 of 17 (35%) patients who progressed to diabetes but in only 1 of 20 (5%) nondiabetic patients. Antibodies to 37k antigen were only found in individuals who became diabetic, including two of three SMS patients with diabetes. More strikingly, antibodies to 37k antigen were detected in 90% of patients in our polyendocrine cohort who developed acute-onset IDDM, whereas all those in whom IDDM was preceded by a prolonged period of NIDDM were negative. Thus, antibodies to 37k antigen identify heterogeneity in the rate of progression to IDDM and are apparently markers of acute-onset diabetes in this population. In support of this, antibodies to 37k antigen were shown to be associated with rapid progression to IDDM in [ICA.sup.+] relatives of patients with IDDM (28).

Studies in patients with polyendocrinopathy have dissected two antibody specificities closely linked to IDDM development: whole islet ICAs and antibodies to 37k antigen. As previously shown in identical twins (16), levels of antibodies to 37k antigen were significantly correlated with ICA titers in the group of IDDM patients who lacked other organ-specific antibodies (and who tended to possess whole islet ICAs), suggesting that these antibodies might arise through common mechanisms. GAD antibodies, although disease-associated, appear in a wider population, only a subset of whom develop IDDM. Harrison et al. (29) have demonstrated an inverse correlation between antibody and T-cell responses to GAD in [ICA.sup.+] relatives of diabetic patients and have

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suggested that autoimmunity to GAD might deviate toward either humoral or cellular responses. Because IDDM is likely to be a T-cell-mediated disease, T-cell responses to GAD may be better indicators of progression to diabetes than are levels of antibodies to the antigen. Whether GAD antibodies in the absence of other diabetes-associated antibodies are markers of slow progression to diabetes remains to be substantiated.

The discovery of serological markers with high disease specificity offers the potential to identify individuals at risk for IDDM with increased accuracy. Among several now available, antibodies to 37k antigen seem to be closely associated with rapid progression to disease. Further characterization of the 37k antigen, including molecular cloning and expression of recombinant protein, should facilitate development of simpler screening assays for the detection of these antibodies. Large-scale screening studies in the general population are required to establish the true predictive value of different antibody specificities. With the availability of accurate markers for disease development, application of immune intervention protocols can be considered as a means of preventing IDDM.

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REFERENCES

(1.)Bottazzo GF, Florin-Christensen A, Doniach D: Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet 2:1279--1283, 1974

(2.)Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, De Camilli P: Identification of the 64k autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347:151--156, 1990

(3.)Christie MR, Brown TJ, Cassidy D: Binding of antibodies in sera from type I (insulin-dependent) diabetic patients to glutamate decarboxylase from rat tissues: evidence for antigenic and non-antigenic forms of the enzyme. Diabetologia 35:380--384, 1992

(4.)Kaufman DL, Erlander MG, Clare-Salzer M, Atkinson MA, Maclaren NK, Tobin AJ: Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. J Clin Invest 89:283--292, 1992

(5.)Karlsen AE, Hagopian WA, Petersen JS, Boel E, Dyrberg T, Grubin CE, Michelsen BK, Madsen OD, Lernmark A: Recombinant glutamic acid decarboxylase (representing the single isoform expressed in human islets) detects IDDM-associated 64,000-[M.sub.r] autoantibodies. Diabetes 41: 1355--1359, 1992

(6.)Rowley MJ, Mackay IR, Chen Q-Y, Knowles WJ, Zimmet PZ: Antibodies to glutamic acid decarboxylase discriminate major types of diabetes mellitus. Diabetes 41:548--551, 1992

(7.)De Aizpurua HJ, Wilson Y, Harrison LC: Glutamic acid decarboxylase autoantibodies in preclinical insulin-dependent diabetes. Proc Natl Acad Sci USA 89:9841--9845, 1992

(8.)Atkinson MA, Kaufman DL, Campbell L, Gibbs KA, Shah SC, Bu D-F, Erlander MG, Tobin AJ, Maclaren NK: Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. Lancet 339:458--459, 1992

(9.)Solimena M, Folli F, Denis-Donini S, Comi GC, Pozza G, De Camilli P, Vicari AM: Autoantibodies to glutamic acid decarboxylase in a patient with stiff-man syndrome, epilepsy and type I diabetes mellitus. N Engl J Med 318:1012--1020, 1988

(10.)Solimena M, Folli F, Aparisi G, Pozza G, De Camilli P: Autoantibodies to GABA-ergic neurons and pancreatic beta cells in stiff-man syndrome. N Engl J Med 322:1555--1560, 1990

(11.)Genovese S, Bonifacio E, McNally JM, Dean BM, Wagner R, Bosi E, Gale EAM, Bottazzo GF: Distinct cytoplasmic antibodies with different risks for type I

Antibodies to islet 37k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity.

(insulin-dependent) diabetes mellitus. Diabetologia 35:385--388, 1992

(12.)Bosi E, Becker F, Bonifacio E, Wagner R, Collins P, Gale EAM, Bottazzo GF: Progression to type I diabetes in autoimmune endocrine patients with islet cell antibodies. Diabetes 40:977--984, 1991

(13.)Palmer JP: Insulin autoantibodies: their role in the pathogenesis of IDDM. Diabetes Metab Rev 3:1005--1015, 1987

(14.)Christie MR, Vohra G, Champagne P, Daneman D, Delovitch TL: Distinct antibody specificities to a 64-kD islet cell antigen in type I diabetes as revealed by trypsin treatment. J Exp Med 172:789--795, 1990

(15.)Christie MR, Hollands JA, Brown TJ, Michelsen BK, Delovitch TL: Detection of pancreatic islet 64,000 Mr autoantigens distinct from glutamate decarboxylase. J Clin Invest 92:240--248, 1993

(16.)Christie MR, Tun RYM, Lo SSS, Cassidy D, Brown TJ, Hollands J, Shattock M, Bottazzo GF, Leslie RDG: Antibodies to glutamic acid decarboxylase and tryptic fragments of islet 64kD antigen as distinct markers for the development of insulin-dependent diabetes: studies with identical twins. Diabetes 41:782--787, 1992

(17.)Bonifacio E, Bingley PJ, Shattock M, Dean BM, Dunger D, Gale EAM, Bottazzo GF: Quantification of islet cell antibodies and prediction of insulin-dependent diabetes. Lancet 335:147--149, 1990

(18.)Kurtz AB, DiSilvio L, Bosi E: The determination of detection limits for insulin antibody assays. Diabetologia 31:395--399, 1988

(19.)Scherbaum WA, Mirakian R, Pujol-Borrell R, Dean BM, Bottazzo GF: Immunocytochemistry in the study of organ-specific autoimmune diseases. In Immunocytochemistry: Modern Methods and Applications. 2nd ed. Polak JM, Van Noorden S, Eds. Bristol, U.K., Wright, 1986, p. 456--476

(20.)Bottazzo GF: On the honey disease: a dialogue with Socrates. Diabetes 42:778--800, 1993

(21.)Barnett AH, Eff C, Leslie RDG, Pyke DA: Diabetes in identical twins: a study of 200 pairs. Diabetologia 20:87--93, 1981

(22.)Gianani R, Pugliesi A, Bonner-Weir S, Shiffrin AJ, Soeldner JS, Erlich H, Awdeh Z, Alper CA, Jackson RA, Eisenbarth GS: Prognostically significant heterogeneity of cytoplasmic islet cell antibodies in relatives of patients with type I diabetes. Diabetes 41:347--353, 1992

(23.)Betterle C, Presotto F, Moro L, Slack RS, Zanette F, Zanchetta R: Islet cell and insulin autoantibodies in organ-specific autoimmune patients: their behaviour and predictive value for the development of type I (insulindependent) diabetes mellitus: a 10-year follow up study. Diabetologia 30:292--297, 1987

(24.)Martino GV, Tappaz ML, Braghi S, Dozio N, Canal N, Pozza G, Bottazzo GF, Grimaldi LME, Bosi E: Autoantibodies to glutamic acid decarboxylase (GAD) detected by an immuno-trapping enzyme activity assay: relation to insulin-dependent diabetes mellitus and islet cell antibodies. J Autoimmun 4:915--923, 1991

(25.)Wulffrat NM, Drexhage HA, Bottazzo GF: Autoimmune aspects of Addison's disease. In The Adrenal Gland. 2nd ed. James VHT, Ed. New York, Raven, 1992, p. 263--288

(26.)Velloso LA, Winqvist O, Gustafsson J, Kampe O, Karlsson FA: Autoantibodies against a novel 51kDa islet antigen and glutamate decarboxylase isoforms in autoimmune polyendocrine syndrome type I. Diabetologia 37:61--69, 1994

(27.)Wagner R, McNally JM, Bonifacio E, Genovese S, Foulis AK, McGill M, Christie MR, Betterle C, Bosi E, Bottazzo GF: Lack of immunohistological changes in the islets of nondiabetic autoimmune polyendocrine patients with long-standing [beta]-selective GAD-specific islet cell antibodies. Diabetes 43:851--856, 1994

(28.)Bingley PJ, Christie MR, Bonfante R, Bonifacio E, Shattock M, Fonte M, Gale EAM, Bottazzo GF: Combined analysis of autoantibodies enhances prediction of IDDM in islet cell antibody (ICA) positive relatives. Autoimmunity 15 (Suppl.):67 1993

(29.)Harrison LC, Honeyman MC, De Aizpurua HJ, Schmidli RS, Colman PG, Tait BD, Cram DS: Inverse relationship between humoral and cellular immunity to glutamic acid decarboxylase in subjects at risk of insulindependent diabetes. Lancet 341:1365--1369, 1993

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IDDM, insulin-dependent diabetes mellitus; ICA, islet cell antibody; GAD, glutamic acid decarboxylase; SMS, stiff-man syndrome; NIDDM, non-insulindependent diabetes mellitus; JDF U, Juvenile Diabetes Foundation units; SLE, systemic lupus erythematosus; IFL, immunofluorescence; IAA, insulin autoantibody; TG-Ab, antibodies to thyroglobulin; TM-Ab, antibodies to thyroid microsomes; GPC-Ab, antibodies to gastric parietal cells; GAD-Ab, antibodies to GAD; 50k-Ab, antibodies to the 50,000 [M.sub.r] fragment of islet [GAD.sub.65]; 37k-Ab, antibodies to 37,000 [M.sub.r] tryptic fragments of islet 37k antigen.

Antibodies to glutamic acid decarboxylase as predictors of insulin-dependent diabetes mellitus before clinical onset of disease

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Summary

We have done a study designed to ascertain the effectiveness of measuring antibodies to glutamic acid decarboxylase (anti-GAD) in predicting insulin-dependent diabetes mellitus (IDDM).

Anti-GAD was measured in prediabetic sera from 151 women aged 20–39 years with newly diagnosed diabetes mellitus who had been identified through a nationwide diabetes register. Multiple serum samples had been collected from these women up to 10 years before the clinical onset of diabetes during their earlier pregnancies. Anti-GAD was measured with a radioimmunoprecipitation assay. Anti-GAD was detected in 82% of 28 women with IDDM, in 36% of 11 women with non-insulin-dependent diabetes mellitus, and in 5% of 112 women with gestational diabetes mellitus. In a random sample of 100 non-diabetic young Finnish women, none had anti-GAD. The sensitivity of the anti-GAD assay for predicting IDDM was 82·1% and the specificity was 100%. The longest time of anti-GAD positivity before clinical onset of IDDM was 10 years. Once positive, anti-GAD levels remained stable and no patients became negative after a positive test during the prediabetic period.

Anti-GAD is a valuable early predictive marker and is associated with a very high risk for development of IDDM.

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Introduction

In insulin-dependent diabetes mellitus (IDDM), pancreatic beta cells are progressively destroyed. Immunological markers such as islet cell antibodies (ICA),[1] insulin autoantibodies (IAA),[2] and antibodies to glutamic acid decarboxylase (anti-GAD)[3–6] are detectable in the sera of patients newly diagnosed with IDDM. Although ICA and IAA can serve as predictive markers for IDDM, the sensitivity and specificity of these antibodies for prediction of IDDM are not good.[7,8] Anti-GAD may be the primary immunological marker in IDDM[9–11]—they are the first to appear in the disease and at the time of diagnosis of IDDM about 75% of patients are positive for anti-GAD.[3–6,12] Anti-GAD are also useful for predicting which patients with apparent non-insulin-dependent diabetes mellitus (NIDDM) at diagnosis will subsequently progress to IDDM.[5,13–15] Loss of tolerance to GAD is probably a step in the development of diabetes in NOD mice,[16,17] and injection of GAD can prevent diabetes in NOD mice. Whether these results are applicable to human beings is unknown.

Data on anti-GAD during the prediabetic period are scarce. Two studies[6,18] reported that about 70–80% of subjects who subsequently developed IDDM were anti-GAD positive before the onset of clinical disease. However, these studies used highly selected patients, mainly siblings or other relatives of IDDM patients. Thus, there have been no population-based prospective data to judge the effectiveness of anti-GAD positivity in predicting IDDM. The period during which anti-GAD may be present before the onset of clinical IDDM is also not known. We have done a study designed to answer these questions.

Patients and methods

Since the beginning of 1992 all newly diagnosed cases of diabetes among subjects aged 15 to 39 years in Finland have been reported to the National Public Health Institute with structured forms. We classified subjects as IDDM if insulin treatment had started by the time the case was reported to the register, or as NIDDM if the patient received diet or oral antidiabetic therapy. The diagnostic criteria for NIDDM were those recommended by WHO. The register recommended the same diagnostic criteria for gestational diabetes mellitus (GDM) as for NIDDM, but since no national criteria for GDM existed at the time of our study we had to rely on criteria applied by local hospitals.

The National Public Health Institute also conducts nationwide screening for conditions such as rubella, hepatitis B antigen, and syphilis antibodies in all pregnant women in Finland. At the antenatal clinic a serum sample is taken from all pregnant women during the first trimester and sent to the National Public Health Institute. After completion of routine screening, residual sera have been stored frozen since 1983.

Of women diagnosed with diabetes in the nationwide diabetes register between January, 1992, and March, 1993, 207 had children. These women were contacted to obtain written consent to use their

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	Number of women studied	Cases positive for anti-GAD		Age (yr)	
		Number (%)	95% CI	Mean	Range
Type of diabetes					
Insulin-dependent	28	23 (82)	63 1–93 9	30	23–39
Non-insulin-dependent	11	4 (34)	10 9–69 2	32	22–39
Gestational: insulin-treated	32	5 (15)	5 3–32 8	32	22–40
Gestational: diet-treated	80	1 (1)	0–6 8	32	20–45
Random population sample	100	0 (0)	0–3 6	36	25–45

Table 1: **Proportion of anti-GAD-positive subjects during the prediabetic period among diabetic women and in a random sample of young women**

sera in the present study. 183 consented, and at least one serum sample obtained during a pregnancy was available for 151 women. Of the 24 women who refused consent, 2 had NIDDM, 7 IDDM, and 15 GDM of whom 2 were treated with insulin. The greatest number of samples from an individual was five from different pregnancies, and the longest period between collection of sera and diagnosis of diabetes was 10 years. We also determined anti-GAD in sera from 100 non-diabetic Finnish women aged 25 to 45 years, a subsample of a cohort randomly selected and investigated for cardiovascular risk factors in 1992.

Anti-GAD was measured by radioimmunoprecipitation with purified porcine brain GAD.[13,19] Concentrations of anti-GAD were measured against a standard reference serum given an arbitrary value of 100 units of activity and reactive sera were given values as a percentage of the reference activity. Based on data from healthy blood donors, the cutoff for positivity in the radioimmunoprecipitation assay was greater than 18 units. The assay as used in the present study does not quantify precisely antibody above 80 units, for which titration is required.[19]

Results

In the sera collected during the prediabetic period, anti-GAD was found in 82% of IDDM patients and in 36% of NIDDM patients who were not treated with insulin and were not pregnant at the time of diagnosis of diabetes (table 1). Of women with GDM, 5% were positive for anti-GAD, but those who had received insulin had a higher prevalence (16%) of anti-GAD than women with GDM treated only with diet (1%).

The probability of correctly identifying women who subsequently developed IDDM by anti-GAD positivity (sensitivity of the test) was 82·1% (23 of 28). The specificity of the assay (probability of correctly identifying non-IDDM women by absence of anti-GAD) was 100% (100 of 100).

Among women who developed IDDM, the proportion of anti-GAD-positive subjects of those tested was over 80% at almost every point in time during the prediabetic period of 1 to 10 years (table 2). Positivity for anti-GAD was very stable, since all positive subjects remained so in subsequent blood samples. Anti-GAD levels varied only slightly on retesting during the prediabetic period. The intra-individual and inter-individual variation in anti-GAD indicated that there was no progressive increase nor decrease in anti-GAD during the prediabetic period.

Time before diagnosis of IDDM (yr)	Cumulative number of subjects tested for first time	Cumulative number of subjects positive for anti-GAD	Percentage of anti-GAD-positive subjects
10	1	1	100 0
9	7	6	85 7
8	8	7	85 7
7	8	7	85 7
6	12	9	75 0
5	15	12	80 0
4	20	17	85 0
3	21	18	85 7
2	25	21	84 0
1	28	23	82 1
0	28	23	82 1

Only the first (most distant from diagnosis of diabetes) anti-GAD test was considered for each subject if several successive blood samples were available

Table 2: **Anti-GAD positivity before diagnosis of diabetes in young women with newly diagnosed IDDM**

Discussion

Our population-based data provide evidence that anti-GAD is a valuable predictive marker of IDDM before the onset of clinical symptoms. As far as is known, there may not be a better marker than anti-GAD for detecting the ongoing process of beta-cell destruction leading to IDDM. The anti-GAD test is simple to perform and standardise and applicable to population sampling. By contrast, the ICA test is difficult to standardise and labour-intensive. Population studies have shown that the positive predictive value of ICA to predict IDDM is only 9–34%.[8] Knowing these shortcomings of ICA testing, we decided not to carry out comparative analyses of ICA and anti-GAD in the present study.

We had the opportunity to test multiple samples and samples collected over a 10-year period before the diagnosis of IDDM. As known from other studies carried out mainly after diagnosis of IDDM,[7,15,18] anti-GAD values do not vary much over time or with age. None of the anti-GAD-positive subjects became negative during the subsequent follow-up in the prediabetic period. Anti-GAD levels did not increase towards the onset of clinical symptoms of IDDM.

Why some IDDM patients remain anti-GAD negative is not known. The test used for anti-GAD is clearly sensitive, since positive samples in our study were repeated and remained positive within narrow margins over several years. There is a known strong genetic component in IDDM and the current view is that only genetically predisposed individuals will get the disease.[20] Studies on anti-GAD have shown that the positivity correlates highly with HLA-DR and DQ phenotypes.[21,22] It would be important to determine whether anti-GAD is genetically determined.

Genetic susceptibility to IDDM and to NIDDM may be located in the HLA region of chromosome 6.[23] Because anti-GAD is found mostly in IDDM patients, but also at lower frequency in NIDDM,[7,13-15] anti-GAD may be a consequence, not the cause of, beta-cell destruction. There is uncertainty in classification of patients into different types of diabetes, as shown by follow-up data from the Swedish nationwide register of diabetes in the population aged 15 to 34 years that showed that 14% of cases originally diagnosed as NIDDM progressed to IDDM and 10% of newly diagnosed cases of diabetes could not be unequivocally classified at the time of diagnosis.[24] It has been proposed that a considerable proportion (about 20%) of NIDDM patients with onset in adult life will slowly develop insulin dependency.[25,26] Previous results have shown that anti-GAD provides the best way to predict which NIDDM patients will later need regular insulin treatment.[5,14,15] The study of anti-GAD in diabetic patients in the Swedish diabetes register for young adults showed that many patients diagnosed with NIDDM were considered positive for "GAD-index" and were later reclassified as having IDDM.[27] One of the probable reasons for the high frequency of anti-GAD positivity in Finnish young women with NIDDM, defined on the basis of not being treated with insulin at the time of or soon after

diagnosis of diabetes, is that they did not develop insulin dependency acutely. We will follow-up these women to see how many will ultimately be classified as having IDDM.

Many women with GDM will later develop frank diabetes, and it has been estimated that about 2% of women with GDM will proceed to IDDM during the subsequent 15 years.[28] In the present study, 5% of all women with GDM were anti-GAD positive, and most of the anti-GAD-positive women with GDM (5 of 6) required insulin treatment during pregnancy. How many of these anti-GAD-positive women will later develop IDDM remains to be seen. Identification of women with GDM who have a high likelihood of developing IDDM is important to allow early intervention to prevent IDDM or its complications. Whether the predictive value of anti-GAD for IDDM is different in women and men is not known. In a study of recent-onset IDDM in childhood in Australia, there was a small sex difference in percentage of anti-GAD positives (females 75%, males 63%; C F Verge, Royal Alexandra Hospital for Children Sydney, Australia, personal communication). There is no evidence to suggest that pregnancy should influence anti-GAD concentrations—in our study anti-GAD-positive women who were retested serially over several pregnancies had stable values of anti-GAD.

The sensitivity and specificity of anti-GAD positivity to detect IDDM in sera from pregnant women were high. Results were sufficiently good for the anti-GAD assay to be used prospectively as a screening test to detect individuals at risk of developing IDDM. On the other hand, before mass screening can be recommended, an effective and acceptable treatment to prevent IDDM should be available. Furthermore, it is important to find out how much beta-cell capacity is left in subjects who are anti-GAD positive. Experimental data indicate that loss of about 80–90% of beta cells is needed before clinical symptoms or diabetes occur.[29] If anti-GAD positivity is present when people are approaching the critical level of beta-cell capacity, anti-GAD positivity may be used for secondary and tertiary prevention of IDDM.

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References

1 Lernmark A, Freedman ZR, Hofmann C, et al. Islet cell surface antibodies in juvenile diabetes mellitus. *N Engl J Med* 1978; 299: 375–80.

2 Palmer JP, Asplin CM, Clemons P, et al. Insulin antibodies in insulin-dependent diabetes before insulin treatment. *Science* 1983; 222: 1337–39.

3 Baekkeskov S, Nellsen JH, Marner B, Bilde T, Ludvigsson J, Lernmark A. Autoantibodies in newly diagnosed diabetic children immunoprecipitate human pancreatic islet cell proteins. *Nature* 1982; 298: 167–69.

4 Christie M, Landin-Olsson M, Sundkvist G, Dahlquist G, Lernmark Å, Baekkeskov S. Antibodies to a M_r 64,000 islet cell protein in Swedish children with newly diagnosed type 1 (insulin-dependent) diabetes. *Diabetologia* 1988; 31: 597–602.

5 Hagopian WA, Karlsen AE, Gottsater A, et al. Quantitative assay using recombinant human islet glutamic acid decarboxylase (GAD65) shows that 65K autoantibody positivity at onset predicts diabetes type. *J Clin Invest* 1993; 91: 368–74.

6 Atkinson MA, Maclaren NK, Scharp DW, Lacy PE, Riley WJ. 64,000 M_r autoantibodies as predictors of insulin-independent diabetes. *Lancet* 1990; 335: 1357–60.

7 Wilkin T, Hoskins PJ, Armitage M, et al. Value of insulin autoantibodies as serum markers for insulin dependent diabetes mellitus. *Lancet* 1985; i: 480–82.

8 Bingley PJ, Bonfacio E, Shattock M, et al. Can islet cell antibodies predict IDDM in the general population. *Diabetes Care* 1993; 16: 45–50.

9 Baekkeskov S, Aanstoot HJ, Christgau S, et al. Identification of the 64K autoantigens in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 1990; 347: 151–56.

10 Reetz A, Solimena M, Matteoli M, Golli F, Takei K, de Camili P. GABA and pancreatic beta-cells: co-localization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. *EMBO J* 1991; 10: 1275–84.

11 Kaufman DL, Erlander MG, Clare-Salzler M, Atkinson MA, Maclaren NK, Tobin AJ. Autoimmunity to two forms of glutamic decarboxylase in insulin-dependent diabetes mellitus. *J Clin Invest* 1992; 89: 283–92.

12 Thivolet CH, Tappaz M, Durand A, Petersen J, Stefanutti A, Chatelain P. Glutamic acid decarboxylase (GAD) autoantibodies are additional predictive markers of type 1 (insulin-dependent) diabetes mellitus in high risk individuals. *Diabetologia* 1992; 35: 570–76.

13 Rowley MJ, Mackay IR, Chen Q-Y, Knowles WJ, Zimmet PZ. Antibodies to glutamic acid decarboxylase discriminate major type of diabetes mellitus. *Diabetes* 1992; 41: 548–51.

14 Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, Mackay IR. Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. *Diabetes* 1993; 42: 359–62.

15 Zimmet PZ, Tuomi T, Mackay IR, Rowley MJ, Knowles W, Cohen M. Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabetic Med* 1994; 11: 299–303

16 Kaufman DL, Clare-Salzler M, Tian J, Forsthuber T, Ting GSP, Robinson P. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 1993; 366: 69–72.

17 Tisch R, Yang XD, Singer SM, Libiau RS, Fugger L, McDevitt HO. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. *Nature* 1993; 366: 72–75.

18 Baekkeskov S, Kristensen JK, Srikanta S, Bruining GJ, Mandrup-Poulsen T, DeBeaufort C. Antibodies to a M_r 64,000 human islet cell antigen precede the clinical onset of insulin dependent diabetes. *J Clin Invest* 1987; 79: 926–34.

19 Chen Q-Y, Rowley MJ, Byrne GC, et al. Antibodies to glutamic acid decarboxylase in Australian children with insulin dependent diabetes mellitus and their first degree relatives. *Pediatric Res* 1993; 34: 785–90.

20 Tuomilehto-Wolf E, Tuomilehto J. HLA antigens in insulin-dependent diabetes mellitus. *Ann Med* 1991; 23: 481–88.

21 Serjeantson SW, Kohonen-Corish MRY, Rowley MJ, Mackay IR, Knowles W, Zimmet P. Antibodies to glutamic acid decarboxylase are associated with HLA-DR genotypes in both Australians and Asians with type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 1992; 35: 996–1001.

22 Serjeantson S, Court J, Mackay IR, et al. HLA-DQ genotypes are associated with autoimmunity to glutamic acid decarboxylase in insulin dependent diabetes mellitus patients. *Hum Immunol* 1993; 38: 97–104.

23 Tuomilehto-Wolf E, Tuomilehto J, Hitman GA, et al. Genetic susceptibility to non-insulin dependent diabetes mellitus and glucose intolerance are located in HLA region. *BMJ* 1993; 307: 155–59.

24 Blohme G, Nyström L, Arnqvist HJ, et al. Male predominance of type 1 (insulin-dependent) diabetes mellitus in young adults: results from a 5-year prospective nationwide study of the 15–34 age group in Sweden. *Diabetologia* 1993; 35: 56–62.

25 Scott RS, Brown LJ. Prevalence and incidence of insulin treated diabetes mellitus in adults in Canterbury, New Zealand. *Diabetic Med* 1991; 8: 1–8.

26 Harris MI, Zimmet P. Classification of diabetes mellitus and other categories of glucose intolerance. In: Keen H, DeFronzo R, Alberti KGMM, Zimmet P, eds. The international textbook of diabetes mellitus. London: Wiley, 1992: 3–18.

27 Landin-Olsson M, Östman J, Lernmark Å, Nystrom I., Schersten B, and the DISS group. ICA, GAD65-AB and C-peptide in the differential diagnosis of diabetes type in young adults. *Diabetologia* 1993; 36 (suppl 1): A99.

28 Catalano PM, Tyzbur ED, Sims EAH. Incidence and significance of islet cell antibodies in women with previous gestational diabetes. *Diabetes Care* 1990; 13: 478–82.

29 Bonnevie-Nielsen V, Steffes MW, Lernmark A. A major loss in the islet mass and β-cell function precedes hyperglycemia in mice given multiple low doses of streptozocin. *Diabetes* 1981; 30: 424–29.

Latent Autoimmune Diabetes Mellitus in Adults (LADA): the Role of Antibodies to Glutamic Acid Decarboxylase in Diagnosis and Prediction of Insulin Dependency

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Type 1 diabetes mellitus in adults may present in a manner similar to that of Type 2 diabetes but with a late development of insulin dependency. We studied 65 patients who presented with 'adult-onset' diabetes after the age of 30 years. Of these patients, 19 required insulin therapy. The insulin-treated patients were significantly younger, their onset of diabetes was at an earlier age, and their postprandial serum C-peptide levels were lower than those of the non-insulin-treated group. Moreover, the insulin-treated subjects had a higher mean concentration of antibodies to glutamic acid decarboxylase (GAD) (66.8 ± 10.2 units) than the patients who did not require insulin (9.9 ± 1.9 units) ($p < 0.001$) and their frequency of anti-GAD positivity was 73.7 % versus 4.3 % ($p < 0.001$). Thus, among patients attending a diabetes clinic, the majority (73.7 %) of subjects who presented with diabetes after 30 years of age and who subsequently required therapy with insulin, actually have the islet cell lesion of Type 1 diabetes which progresses at a slower tempo than in children. We conclude that testing for anti-GAD in adult-onset non-obese diabetic patients should be a routine procedure in order to detect latent insulin-dependency at the earliest possible stage, since this assay can assist in the correct classification of diabetes, and more appropriate therapy.

KEY WORDS **Type 1 diabetes mellitus Insulin-treated diabetes mellitus Anti-GAD C-peptide**

Introduction

The National Diabetes Data Group (NDDG)[1] and the World Health Organization Expert Committee (WHO) on Diabetes Mellitus[2] recommended that the terms 'mature-onset' and 'adult-onset' diabetes mellitus be replaced by non-insulin-dependent diabetes mellitus (or Type 2 diabetes), with subcategories of non-obese and obese. However, Type 2 diabetes is heterogenous,[3] and while a substantial proportion of cases are obese and hyperinsulinaemic,[4] the non-obese patients with Type 2 diabetes often have insulin hyposecretion,[5,6] and treatment fails with oral hypoglycaemic drugs. Accordingly subjects in this non-obese group could actually have Type 1 diabetes but in a slowly evolving form.[7,8] Scott and Brown conducted a population-based study of insulin-treated adult diabetes patients in New Zealand and found that as many as 14.4 % were insulin-treated, and most (83 %) had commenced insulin as a permanent treatment within 12 months of diagnosis.[9] Similarly, the prevalence of Type 1 diabetes among adult diabetic patients in other countries is higher than previously thought.[10,11] The clinical onset of diabetes is usually less acute in adult patients, so leading to an initial diagnosis of Type 2 diabetes,[3] and there is a group of patients, hitherto labelled as 'Type 1 1/2 diabetes',[3] who are non-obese, and initially may be treated as Type 2 diabetes, yet progress rapidly to insulin dependency, within months or a few years of diagnosis.

Patients reported as 'Type 1 1/2 diabetes' show some of the classical features of Type 1 diabetes including weight loss, low C-peptide concentration, an increased frequency of HLA DR3 and DR4, and positive tests for islet cell antibodies (ICA).[2,12,13] Recently, we reported a high frequency of antibodies to glutamic acid decarboxylase (anti-GAD) in these patients[14,15] and Hagopian *et al.* similarly reported that the anti-GAD assay is very useful for their identification.[16] Therefore, 'Type 1 1/2 diabetes' is likely to be a slowly-evolving Type 1 diabetes, and we suggested the name latent autoimmune diabetes in adults (LADA) be used to describe such patients.[15] Since tests for ICA and fasting C-peptide levels, which predict insulin requirement at the time of diagnosis of diabetes,[17] are technically demanding, hard to standardize,[18] and expensive, a simple discriminatory test

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would be a valuable tool. We describe here the results with a radioimmunoprecipitation assay for anti-GAD as a predictor of insulin dependency in a group of Australian adults who presented with diabetes mellitus.

Patients and Methods

The study population consisted of 65 adult patients who had presented to the International Diabetes Institute, Melbourne, with diabetes in adulthood. Physicians were asked to identify subjects who had initially been treated with diet alone or with oral hypoglycaemic agents, but in whom the diagnosis of Type 1 versus Type 2 diabetes was not clear cut on clinical grounds. The selection criteria included ≥ 30 years of age at diagnosis, absence of ketonuria, and with no apparent need for insulin-treatment for at least 6 months after the initial diagnosis.

There were 46 subjects in whom the current treatment remains diet alone or diet and oral hypoglycaemic agent(s) with acceptable metabolic control of diabetes after a mean observation period of 8.6 years, and 19 subjects who subsequently required insulin therapy after a mean interval of 5.8 years and are designated as insulin-treated diabetes mellitus (ITDM).

The clinical and laboratory characteristics including serum C-peptide and anti-GAD levels of these ITDM patients are shown in Table 1. A group of 53 non-diabetic blood donors were used as controls. The decision to commence insulin therapy was made on clinical grounds. Patient selection occurred prior to the measurement of anti-GAD and physician treatment occurred independently of the results. As height measurements were not available on some of the patients, we have had to use weight rather than body mass index as an index of overweight and obesity.

Most of the patients had attended the Institute from the time of diagnosis of diabetes which was made according to the NDDG criteria for the classification of diabetes.[1]

Serum C-peptide Measurements

C-peptide concentrations were available for the majority of the patients studied and were performed by radioimmunoassay (Diagnostic Systems Laboratories, Webster, Texas, USA). The conditions for C-peptide measurements were such that sera were obtained at the time of clinic visits, i.e. postprandial. Under such conditions a C-peptide value $< 1\ \mu g\ ml^{-1}$ specified insulin deficiency.

Autoantibodies to GAD

Antibodies to GAD were measured by a radioimmunoprecipitation assay using radiolabelled porcine brain GAD that was purified by affinity chromatography and which has been reported in detail elsewhere.[14] The results were expressed as units equalling the percentage of radioactivity precipitated by the test sera of that precipitated by the reference serum, which had 100 units (U). The coefficients of variation (CV) for inter-assay and intra-assay replicates for another high positive control serum (mean 120 U) were 9 % ($n = 13$) and 13 % ($n = 9$), respectively, and the inter-assay CV for a low positive control serum (30 U) was 14 % ($n = 13$). We defined the normal range using the 53 blood donor sera which were tested concurrently. The mean was

Table 1. Clinical features and laboratory data of the 19 insulin-treated diabetic patients

Patient number	Age/sex (yr)	Age at onset (yr)	Weight (kg)	C-peptide μg^{-1}	Anti-GAD (units)
1	53 F	40	69.3	0.3	58
2	54 M	44	71.1	0.4	125
3	63 F	59	55.9	2.4	93
4	57 M	48	68.2	0.3	86
5	44 M	37	57.5	0.9	114
6	50 M	42	81.1	1.5	57
7	62 F	52	47.4	0.1	45
8	70 M	55	88.3	0.7	29
9	59 M	37	78.0	0.5	12
10	40 M	40	92.5	4.9	6
11	52 M	52	88.3	–	118
12	80 F	70	76.1	0.1	103
13	55 F	55	56.7	2.1	102
14	49 M	32	81.2	0.3	87
15	56 F	56	62.5	0.4	92
16	64 F	46	48.5	0.3	122
17	68 F	39	66.0	4.0	4
18	64 M	41	63.0	3.0	6
19	47 F	34	103.2	1.0	10

8.2 ± 2.7 U (range 4–18 U). The limit for positivity was set at 18 U, which exceeds the mean + 3 SD for the controls[14].

Statistical Analysis

Results are expressed throughout as mean and standard errors of the mean. The chi-squared (χ^2) test with Yates correction was used to determine statistical significances of differences between group frequencies, and Student's *t*-test was used to compare means of levels of anti-GAD where appropriate.

Results

The ITDM group differed from the Type 2 diabetes group according to a number of features (Table 2), including a lower mean age ($p < 0.02$), a lower age at onset of diabetes ($p < 0.002$), a lower mean body weight, and a lower mean serum C-peptide concentration ($p < 0.001$). Moreover, the mean level of anti-GAD was 66.8 ± 10.2 units, significantly higher than for the Type 2 diabetes group 9.9 ± 1.9 units ($p < 0.001$), and respective frequencies of positive tests for anti-GAD were 74 % (14/19) versus 4.3 % (2/46).

Attributes of subjects who gave positive or negative tests for anti-GAD are shown in Table 3; distribution of values for these indices is similar to that for ITDM and Type 2 diabetes groups, respectively. In regard to therapy, 87.5 % of the patients who gave a positive test for anti-GAD were receiving treatment with insulin, whereas 81.6 % of the anti-GAD negative patients were treated with dietary modification or oral hypoglycaemic drugs. Of two patients with Type 2 diabetes and a high level of anti-GAD, control of hyperglycaemia is poor (data not shown); one of the two has a sister with Type 1 diabetes.

Discussion

This study confirms recent findings by us[15,19] and Hagopian *et al.*[16] that a substantial proportion of patients, perhaps up to 20 % with an onset of diabetes in adult life have a slow onset type of Type 1 diabetes with automimmune features: we have called this latent autoimmune diabetes of adults. The natural history of LADA is predictable on clinical grounds and from results of ICA and C-peptide[12,16] but the availability of a simple laboratory assay for anti-GAD provides for an easy discrimination in most of these subjects when the information can be used for clinical management, and could allow in future for the use of immune-modulation therapy to preserve insulin secretion. Moreover, these findings confirm that a positive anti-GAD almost invariably means that the patient will require insulin.[15] We can emphasize that testing for anti-GAD is superior to the traditional ICA test in distinguishing patients with LADA and, notably, some of the patients in this study had high levels of anti-GAD before their final decompensation to insulin-dependency, when they were in a 'pre-insulin deficiency' stage. Baekkeskov *et al.* found that over 80 % of subjects who subsequently developed Type 1 diabetes were anti-GAD positive several years prior to the clinical onset of the disease,[20] a situation we have recently confirmed with our radio-immunoprecipitation assay (Zimmet *et al.*, unpublished).

Type 1 diabetes has attributes consistent with it being an autoimmune disease[21] although there are clearly some patients with Type 1 diabetes with insulin deficiency from other causes. Observations from our laboratory based on various sources of patients[14,22] indicate that over 70 % of newly diagnosed Type 1 diabetes subjects have high titres of anti-GAD, and patients with this form of diabetes have other features consistent with autoimmunity including ICA and autoantibodies to insulin, and specific associations with MHC (HLA) alleles.[3,21] Apart from these features, the autoimmune process evolves to total islet beta-cell destruction since Type 1 diabetes is characterized by absolute insulin deficiency and life-long insulin-dependency.

Type 2 diabetes comprises a heterogenous group of disorders[3,23] including the entities of non-obese and obese Type 2 diabetes, maturity-onset diabetes in youth, certain rare genetic syndromes, and subjects with so-

Table 2. Comparison of characteristics of patients with insulin-treated diabetes mellitus (ITDM) and those with Type 2 diabetes

	ITDM	Type 2 diabetes	*p*
n	19	46	
Age (yr)	57.2 ± 2.2	63.5 ± 1.4	0.02
Age of onset (yr)	46.3 ± 2.3	55.3 ± 1.4	0.002
Duration of diabetes (yr)	11.0 ± 1.9	8.6 ± 0.9	NS
Duration of insulin treatment (yr)	5.2 ± 1.2	–	–
Weight (kg)	71.3 ± 3.5	78.6 ± 2.3	NS
C-peptide (μg ml^{-1})	1.3 ± 0.36	4.6 ± 0.39	< 0.001
Anti-GAD (units)	66.8 ± 10.2	9.9 ± 1.9	< 0.001
Frequency anti-GAD positivity	73.7 %	4.3 %	< 0.001

Results as mean ±SEM.

Table 3. Characteristics of patients with 'adult-onset' diabetes in relation to anti-GAD status

Parameter	Anti-GAD status		p
	GAD '+'	GAD '−'	
Number	16	49	
Age (yr)	56.6 ± 2.4	63.3 ± 1.37	0.021
Age of onset (yr)	48.8 ± 2.4	54.0 ± 1.5	NS
Duration of diabetes (yr)	7.8 ± 1.5	9.7 ± 1.0	NS
Weight (kg)	69.1 ± 3.3	78.9 ± 2.3	0.021
C-peptide (μg ml^{-1})	1.3 ± 0.4	4.3 ± 0.4	< 0.001
Anti-GAD (units)	84.3 ± 8.0	7.7 ± 0.5	< 0.001
Treatment modality (%)			
Diet	0.0	8.2	< 0.001
Oral agents	12.5	81.6	< 0.001
Insulin	87.5	10.2	< 0.001

Results as mean ±SEM.

called 'Type 1 1/2 diabetes', now designated by us as latent autoimmune diabetes in adults, but this condition should be clearly identified with Type 1 diabetes in any forthcoming revision of the classification of diabetes. Although hyperinsulinaemia is a characteristic of Type 2 diabetes,[4] there are cases of Type 2 diabetes with genuine insulin deficiency,[5] many are in the non-obese category with a beta-cell secretory defect whereas a proportion, perhaps 10–15 %, are in transition to Type 1 diabetes.[9,19] This group can be defined with acceptable sensitivity and specificity by the assay for anti-GAD.[15] It is now clear that Type 1 diabetes can occur after a prolonged period of what is clinically accepted as Type 2 diabetes.[9,12,13] Such patients present with typical clinical features of Type 2 diabetes, and can be treated with diet alone, or with diet and oral hypoglycaemic drugs even for several years before progressing to insulin dependency. This subgroup has distinctive immunological and metabolic features that distinguish its members from those with Type 2 diabetes.[8,12,13,15,16] Groop and colleagues[12] suggested that these cases were 'latent' Type 1 diabetes, as judged by persistent ICA, progressive loss of beta-cells and a high frequency of thyrogastic autoimmunity. Furthermore, there was an excess frequency of HLA-DR3 and -DR4[13] confirming an earlier report by Di Mario *et al.*[24] This group clearly cannot be defined by the current NDDG and WHO classifications and will need to be identified as part of the spectrum of Type 1 diabetes if further studies confirm our present findings and the three other recent reports showing a high prevalence of antibodies to GAD.[15,16,19] The clinical implications of LADA include the appreciation that insulin therapy is needed at an early stage of the disease to preserve beta-cell function and achieve better metabolic control so possibly reducing the likelihood of microvascular consequences of the diabetes.[25]

It seems clear that Type 1 diabetes can occur in all age groups, with a higher prevalence and incidence in middle and older age groups in Europids than hitherto suspected[9–11] and this may particularly be the case in Asian populations where Type 1 diabetes in childhood is rare.[3,7] We have shown that testing for anti-GAD is superior to the traditionally used ICA in distinguishing those adult onset diabetics who fit into the Type 1 diabetes category.[15] The availability of the anti-GAD assay allows the identification of 'adult-onset' diabetic persons who require insulin therapy and also permits the preclinical diagnosis of Type 1 diabetes both in childhood and adult life. This raises the potential for primary prevention of Type 1 diabetes throughout the whole age range as safer immunotherapy interventions become available.[25]

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References

1. National Diabetes Data Group. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *Diabetes* 1979; **28**: 1039–1057.
2. World Health Organization Expert Committee on Diabetes Mellitus. *Second Report.* Technical Report Series 646. Geneva: WHO, 1980.
3. Harris MI, Zimmet P. Classification of diabetes mellitus and other categories of glucose intolerance. In: Keen H, DeFronzo R, Alberti KGMM, Zimmet P, eds. *The International Textbook of Diabetes Mellitus.* London: Wiley, 1992: 3–18.
4. Zimmet P. Kelly West Lecture 1991. Challenges in

diabetes epidemiology—from West to the rest. *Diabetes Care* 1992: **15**: 232–252.
5. Temple RC, Carrington CA, Luzio SD, Owens DR, Schneider AE, Sobey WJ, *et al*. Insulin deficiency in Type 2 diabetes. *Lancet* 1989; **1**: 293–295.
6. Gjessing HJ, Matzen LE, Faber OK, Froland A. Fasting plasma C-peptide, glucagon stimulated C-peptide and urinary C-peptide in relation to clinical type of diabetes. *Diabetologia* 1989; **32**: 305–311.
7. Zimmet PZ, Rowley MJ, Mackay IR, Knowles WJ, Chen Q-Y, Chapman LH, *et al*. The ethnic distribution of antibodies to glutamic acid decarboxylase: Presence and levels in insulin-dependent diabetes mellitus in Europid and Asian subjects. *J Diabetes and Its Complications* 1993; **7**: 1–7.
8. Groop L, Pelkonen R, Roakimiej S, Bottazzo G, Doniach D. Secondary failure treatment with oral antidiabetic agents in non-insulin-dependent diabetes. *Diabetes Care* 1986; **9**: 129–132.
9. Scott RS, Brown LJ. Prevalence and incidence of insulin-treated diabetes mellitus in adults in Canterbury, New Zealand. *Diabetic Med* 1991; **8**: 1–8.
10. Tuomilehto J, Nissinen A, Kivela SL, Pekkanen J, Kaarsalo E, Wolf E, *et al*. Prevalence of diabetes mellitus in elderly men aged 65 to 84 years in Eastern and Western Finland. *Diabetologia* 1986; **29**: 611–615.
11. Libman I, Songer T, LaPorte R. How many people in the USA have IDDM? *Diabetes Care* 1993; **16**: 841–842.
12. Groop L, Bottazzo G, Doniach D. Islet cell antibodies identify latent type I diabetes in patients aged 35–75 at diagnosis. *Diabetes* 1986; **35**: 237–241.
13. Groop L., Miettinen A, Groop P-H, Meri S, Koskimies S, Bottazzo GF. Organ-specific autoimmunity and HLA-DR antigens as markers for beta-cell destruction in patients with type II diabetes. *Diabetes* 1988; **37**: 99–103.
14. Rowley MJ, Mackay IR, Chen Q-Y, Knowles WJ, Zimmet PZ. Antibodies to glutamic acid decarboxylase discriminate major types of diabetes mellitus. *Diabetes* 1992; **41**: 548–551.
15. Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, Mackay IR. Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. *Diabetes* 1993; **42**: 359–362.
16. Hagopian WA, Karlsen AE, Gottsater A, Landin-Olsson M, Grubin CE, Sundkvist G, *et al*. Quantitative assay using recombinant human islet glutamic acid decarboxylase (GAD65) shows that 64K autoantibody positivity at onset predicts diabetes type. *J Clin Invest* 1993; **91**: 368–374.
17. Landin-Olsson M, Nilsson KO, Lernmark Å, Sundkvist G. Islet cell antibodies and fasting C-peptide predict insulin requirement at diagnosis of diabetes mellitus. *Diabetologia* 1990; **33**: 561–568.
18. Palmer JP, McCulloch DK. Prediction and prevention of IDDM—1991. *Diabetes* 1991; **40**: 943–947.
19. Scott R, Willis J, Brown L, Forbes L, Schmidli R, Zimmet P, *et al*. Antibodies to glutamic acid decarboxylase (GAD) predict insulin-deficiency in adult onset diabetes mellitus. *Diabetes* 1993 (suppl 1); **42**: 220A.
20. Baekkeskov S, Kristensen JK, Srikanta S, Bruining GJ, Mandrup-Poulsen T, DeBeaufort C. Antibodies to a M 64,000 human islet cell antigen precede the clinical onset of insulin dependent diabetes. *J Clin Invest* 1987; **79**: 926–934.
21. Eisenbarth GS. Type I diabetes mellitus. A chronic autoimmune diseae. *N Engl J Med* 1986; **314**: 1360–1368.
22. Chen Q-Y, Byrne GC, Jones T, Tuomi T, Mackay IR, Rowley MJ, *et al*. Antibodies to glutamic acid decarboxylase in Australian children with insulin dependent diabetes mellitus and their first degree relatives. *Pediatric Res* 1993 (submitted).
23. Zimmet P. Non-insulin-dependent (Type 2) diabetes mellitus—does it really exist? *Diabetic Med* 1989; **6**: 728–735.
24. Di Mario, Irvine WJ, Borsey DO, Kyner JL, Weston J, Galfo C. Immune abnormalities in diabetic patients not requiring insulin at diagnosis. *Diabetologia* 1983; **25**: 392–396.
25. Skyler JS, Marks JB. Immune intervention in type 1 diabetes mellitus. *Diabetes Rev* 1993; **1**: 15–42.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/660,924	09/12/2003	Paul P. Latta	LATTA.002A	7335

20995 7590 05/18/2005

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EXAMINER
BELYAVSKYI, MICHAIL A

ART UNIT	PAPER NUMBER
1644	

DATE MAILED: 05/18/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/660,924	LATTA, PAUL P.	
	Examiner	Art Unit	
	Michail A. Belyavskyi	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *02 May 2005*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *2-9* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *2-9* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

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RESPONSE TO APPLICANT'S AMENDMENT

1. Applicant's amendment, filed 05/02/05 is acknowledged.

Claims 2-9 are pending.

Claims 2-9 are under consideration in the instant application.

2. The title of the invention is not descriptive. A new title is required that is clearly indicative of the invention *to which the claims are directed.*

Applicant's arguments, filed 05/02/05 have been fully considered, but have not been found convincing.

Applicant asserts that the title accurately reflects the claimed invention.

Contrary to Applicant's assertion, it is noted that the amended claims of the instant application reads on a method of preventing onset of Type I diabetes. The current title of the application reads on prevention of any type of diabetes.

3. The rejection of: (i) claims 2-5 and 7-9 under 35 U.S.C. 102(e) as being anticipated by US Patent 6,703,017 or by US Patent 5,425764 and (ii) claims 2-9 under 35 U.S.C. 103(a) as being unpatentable over US Patent 6,703,017 or by US Patent 5,425764 each and in view of US Patent 5,529,914 are hereby withdrawn in view of the amendment to claim 1. However, said rejections will be re-introduced when the **new matter** (wherein said dose is at least one order of magnitude less that that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes) is deleted from claim 1.

In view of the amendment, filed 05/02/05 the following rejections remain:

4. The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

5. Claims 2-9 are rejected under 35 U.S.C. 112, first paragraph, because the specification, does not reasonably provide **enablement** for a method of preventing onset of Type I diabetes in a mammal , comprising implanting a dose of insulin-producing cells encapsulated in a biologically compatible membrane_wherein said dose is at least one order of magnitude less that that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes , for the same reasons set forth in the previous Office Action, mailed on 12/16/05.

Applicant's arguments, filed 05/02/05 have been fully considered, but have not been found convincing.

Applicant asserts that: (i) US PTO has previously accepted the predictability of the results of prevention of diabetes in nod mice and allowed US Patent 6,841,152; (ii) Second Declaration by Dr. Scharp under 37 CFR 1.132 states that the NOD mouse is the only animal model for human autoimmune, type I diabetes because it is the only available model reasonable predictive of human disease; (iii) NOD mouse model is the standart animal model for conducting research on type I diabetes and one skill in the art would accept the NOD model as reasonable correlating to the condition in human.

Contrary to Applicant's assertion, it is noted the claims of the issue patent US 6,841,152 recites method of protecting against the development of autoimmune diabetes, not method of preventing. Moreover, it is well settled that whether similar claims have been allowed to others is immaterial. See In re Giolito, 530 F.2d 397, 188 USPQ 645 (CCPA 1976) and Ex parte Balzarini 21 USPQ2d 1892, 1897 (BPAI 1991). Moreover, as stated In re Borkowski, 505 F2d 713,718,184 USPQ29,33 (CCPA 1974), "The Paten Office must have the flexibility to reconside and correct prior decisions that may find to have been in error". In a similar context, the court in Fessenden v.Coe, 38 USPQ 516,521 (CADC 1938) stated that '[t]wo wrongs cannot make a right."

With regards to the second declaration of Dr. Scharp under 37 CFR 1.132. The examiner disagree with the statement that NOD mice model is the only rodent model of type I diabetes. Atkinson et al.,(Nature, 1999, V.5, pages 601-604) teach the advantages of BioBreeding rat as a model of type I diabetes (see entire document, page 603 in particular). Moreover, the data presented in the second declaration of Dr. Scharp under 37 CFR 1.132 clearly indicated that using NOD mice as a model, 40% of the treated animals develop diabetes. In other words, even in NOD animal model in 40 % of the animals the onset of diabetes has not been prevented using the claimed method.

With regards to the issue that NOD model correlating with the condition in human.

Atkinson et al. ,(Nature, 1999, V.5, pages 601-604) teach that in addition to certain NOD strain-specific characteristics that distinguish these mice from humans at risk for type I diabetes important genus-specific features distinguish the murine diabetes as well, such as resistance to ketoacidosis or the absence of the murine homolog of HLA-DR molecules on APC. Investigators have not always considered that. Unfortunately, in a genetically heterogeneous human population containing individuals at high risk of type I diabetes development, there is little evidence that many of them would have a comparable set of immune deficiencies that prove as malleable. In NOD mice, type 1 diabetes development is well-choreographed. In contrast, the natural history of type 1 diabetes in human is such that the age of disease onset is extremely broad; symptoms occur at any time from the first years of life to well beyond 50 years of age. It is clear that the genus-unique and strain-specific aspects of diabetes in NOD mice must be fully understand and appreciated if we are to know which therapeutic protocols are reasonable to extrapolate to humans and which are not. Exploitation of the NOD genome for clinical research is yet to be done (see pages 602, 603 and 604 in particular). Moreover, as has been stated in the previous Office Action, the specification only discloses the effects of the implanting of insulin-producing cells on the level of blood glucose using streptozotocin-induced diabeties in murine experimental model. (See Examples 1-2 in particular). Examples 3-7 in the instant Specification are prophetic examples that indicate what the inventor thinks might happen in the experiments which have not actually been performed. The specification does not adequately teach how to effectively prevent onset of type I diabetes in mammal predisposed to type I diabetes , comprising implanting insulin-producing cells encapsulated in a biologically-compatible membrane. Knip M (Acta Pediatr. Suppl., 1998, V.452, pages 54-62) teaches that currently the state of the art is that successful prevention of type I diabetes has at least two precondition. First, one must be able to identify individuals at increase risk for progression to type I diabetes and second, must have an intervention modality with less severe adverse effects than those associated with disease itself. Total eradication of clinical type I diabetes cannot be expected in the next century, as it is probable that a combination of different interventions will be needed to achieve an optimal effect (see entire document, page 60 in particular). Mestas et al (J. of Immunology, 2004, 172, pages 2731-238) teach that there exist significant differences between mice and humans in immune system development, activating and response to challenge in both the innate and adaptive arms. As therapies for human diseases become ever more sophisticated and specifically targeted it becomes increasing important to understand the potential limitations of extrapolating data from mice to humans. The literature is littered with the examples of therapies that work well in mice but fail to provide similar efficacy in humans. Teuveson et al., (Immun. Review 1993, N136, pages 101-107) teach that one problem with rodent models of transplantation is that rejection is easily overcome in said models in comparison to the difficulty of overcoming allograft rejection in human (see page 100 in particular). Teuveson et al., further teach that " however today's small animal models seem to be insufficient to produce data for clinical decision-making" and further raises doubt as to whether large animal models can be applied to clinical situations, due to species-specific reactions to treatment (see page 101 in particular). Feldman et al (Transplant. Proc. 1998, 30, 4126-4127) teach that "while it is not difficult to study the pathogenesis of animal models of disease, there

are multiple constraints on analyses of the pathogenesis of human disease, leading to interesting dilemmas such as how much can we rely on and extrapolate from animal models in disease". In addition, Cochlovius et al (Modern Drug Discovery, 2003, pages 33-38) teach that in contrast to in vitro models, and partly animal-human xenograft systems, tissue cells in vivo seems to express molecules for defense against cellular immune systems as well as against complement. Although these defense mechanisms are still poorly understood, they provide some hints as to why many potential therapeutics perform marvelously in vitro but a fairly high portion of them still fail *in vivo*.

Substantiating evidence may be in the form of animal tests, which constitute recognized screening procedures with clear relevance to efficacy in humans. See Ex parte Krepelka, 231 USPQ 746 (Board of Patent Appeals and Interferences 1986) and cases cited therein. Ex parte Maas, 9 USPQ2d 1746. However, as has been discussed supra, the state of the art is that it is unpredictable form the *in vivo* murine data using NOD model disclosed in the specification as whether the instant invention can be used for the *in vivo* preventing onset of type I diabetes in mammals including human. Therefore, it is not clear that the skilled artisan could predict the efficacy of a method of preventing onset of type I diabetes in mammal predisposed to type I diabetes , comprising implanting insulin-producing cells encapsulated in a biologically-compatible membrane. Thus in the absence of working examples or detailed guidance in the specification, the intended uses of the claimed method of preventing onset of Type I diabetes in any mammal, including human are fraught with uncertainties.

Thus, Applicant has not provided sufficient guidance to enable one skill in the art to use claimed method of preventing onset of type I diabetes in mammal predisposed to type I diabetes , comprising implanting insulin-producing cells encapsulated in a biologically-compatible membrane in manner reasonably correlated with the scope of the claims. The scope of the claims must bear a reasonable correlation with the scope of enablement. *In re Fisher*, 166 USPQ 18(CCPA 1970) indicates that the more unpredictable an area is, the more specific enablement is necessary in order to satisfy the statute.

In view of the quantity of experimentation necessary, the unpredictability of the art, the lack of sufficient guidance in the specification, the limited working examples, and the limited amount of direction provided given the breadth of the claims, it would take undue trials and errors to practice the claimed invention.

The following new ground of rejection is necessitated by the amendment filed 05/02/05

6. The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

7. Claims 2-9 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. **This is a New Matter rejection**.

" ... wherein said dose is at least one order of magnitude less that that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes" claimed in claim 1 represent a departure from the specification. The passages pointed by the applicant do not provide a clear support for the " ...wherein said dose is at least one order of magnitude less that that necessary to achieve normoglycemia in a mammal of the same species with type I diabetes. The specification and the claims as originally field only support administering a tolerizing dose of insulin-producing cells encapsulated in a biologically-compatible membrane. The passage pointed by Applicant only generally disclosed that curative dose is between one and two orders of magnitude greater than the tolerizing dose.

7. No claim is allowed

8. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

9. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michail Belyavskyi whose telephone number is 571/ 272-0840 The examiner can normally be reached Monday through Friday from 9:00 AM to 5:30 PM. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan can be reached on 571/ 272-0841 .

The fax number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Michail Belyavskyi, Ph.D.
Patent Examiner
Technology Center 1600
May 13, 2005

CHRISTINA CHAN
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

PTO/SB/08 Equivalent

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Multiple sheets used when necessary)

SHEET 1 OF 1

Application No.	10/660,924
Filing Date	September 12, 2003
First Named Inventor	Latta, Paul P.
Art Unit	1644
Examiner	Belyavskyi, Michail A.
Attorney Docket No.	LATTA.002C3

U.S. PATENT DOCUMENTS

Examiner Initials	Cite No.	Document Number *Number - Kind Code (if known)* Example: 1,234,567 B1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials	Cite No.	Foreign Patent Document *Country Code-Number-Kind Code* Example: JP 1234567 A1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear	T[1]

NON PATENT LITERATURE DOCUMENTS

Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[1]
MB	1	BINGLEY, P.J. et al. (1994) "Combined analysis of autoantibodies improves production of IDDM in islet cell antibody-positive relatives" Diabetes 43:1304(7).	
	2	BONIFACIO, E. et al. (1995) "Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity" Diabetologia 38:816-822.	
	3	CHRISTIE, M.R. et al. (1994) "Antibodies is islet 37k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity" Diabetes 43:1254(6).	
	4	LEE, H.C. et al. (1995) "Relationships among 64k autoantibodies, pancreatic beta-cell function, HLA-DR antigens and HLA-DQ genes in patients with insulin-dependent diabetes mellitus in Korea" Korean J. Intern Med. (Abstract only).	
	5	TUOMILEHTO, J. et al. (1994) "Antibodies to glutamic acid decarboxylase as predictors of insulin-dependent diabetes mellitus before clinical onset of disease" Lancet 343:1383-1385.	
	6	ZIMMET, P.Z. et al. (1994) "Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency" Diabetic Medicine 11:299-303.	
MB	7	ZIMMET, P.Z. et al. (1994) "Autoantibodies to glutamic acid decarboxylase and insulin in islet cell antibody positive presymptomatic type 1 diabetes mellitus: frequency and segregation by age and gender" Diabetic Medicine 11:866-871.	

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042905

Examiner Signature	5/4/05	Date Considered	

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

T[1] - Place a check mark in this area when an English language Translation is attached.

FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. LATTA.002C3	APPLICATION NO. 10/680,924
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (USE SEVERAL SHEETS IF NECESSARY)	APPLICANT PAUL P. LATTA	
	FILING DATE September 12, 2003	GROUP 1632

U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE (IF APPROPRIATE)
MB	1.	4,298,002	11/03/81	RONEL et al.			
	2.	4,353,888	10/12/82	SEFTON			
	3.	4,378,016	03/29/83	LOEB			
	4.	4,673,566	06/16/87	GOOSEN et al.			
	5.	4,689,293	08/25/87	GOOSEN et al.			
	6.	4,696,286	09/29/87	COCHRUM			
	7.	4,806,355	02/21/89	GOOSEN et al.			
	8.	4,892,538	01/09/90	AEBISCHER et al.			
	9.	4,902,295	02/20/90	WALTHALL et al.			
	10.	4,997,443	03/05/91	WALTHALL et al.			
	11.	5,182,111	01/26/93	AEBISCHER et al.			
	12.	5,262,044	11/16/93	BAE et al.			
	13.	5,290,684	03/01/94	KELLY			
	14.	5,529,914	06/25/96	HUBBELL et al.			
MB	15.	5,425,764	08/20/95	FOURNIER et al.			

FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
MB	16.	A2 0,147,939	10/07/85	EPO				
	17.	A1 2,034,641	28/05/92	CANADA				
	18.	WO 92/19195	12/11/92	PCT				
	19.	WO 95/03062	02/02/95	PCT				
MB	20.	0 536 807 A1	04/02/87	EP				

EXAMINER INITIAL		OTHER DOCUMENTS (INCLUDING AUTHOR, TITLE, DATE, PERTINENT PAGES, ETC.)
MB	21.	Aebischer, P. et al., "LONG-TERM CROSS-SPECIES BRAIN TRANSPLANTATION OF A POLYMER-ENCAPSULATED DOPAMINE-SECRETING CELL LINE" *Experimental Neurology (1991) 111:269-275*
MB	22.	Aebischer, P. et al., "TRANSPLANTATION OF POLYMER ENCAPSULATED NEUROTRANSMITTER SECRETING CELLS: EFFECT OF THE ENCAPSULATION TECHNIQUE" *Journal of Biomechanical Engineering (1991) 113:178-183*

EXAMINER	DATE CONSIDERED 5/11/05

*EXAMINER: INITIAL IF CITATION CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP 609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED, INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. LATTA.002C3	APPLICATION NO. 10/660,924
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (USE SEVERAL SHEETS IF NECESSARY)	APPLICANT PAUL P. LATTA	
	FILING DATE September 12, 2003	GROUP 1632

EXAMINER INITIAL		OTHER DOCUMENTS (INCLUDING AUTHOR, TITLE, DATE, PERTINENT PAGES, ETC.)
MB	23.	Bartlett, S.T. et al., "COMPOSITE KIDNEY-ISLET TRANSPLANTATION PREVENTS RECURRENT AUTOIMMUNE BETA-CELL DESTRUCTION" *Surgery (1993) 114:211-217*
	24.	Buchser, et al., "IMMUNOISOLATED XENOGENIC CHROMAFFIN CELL THERAPY FOR CHRONIC PAIN. INITIAL CLINICAL EXPERIENCE" *Anesthesiol., (1996) 85:1005-1012*
	25.	Chicheportiche, D. et al., "IN VITRO KINETICS OF INSULIN RELEASE BY MICROENCAPSULATED RAT ISLETS: EFFECT OF THE SIZE OF THE MICROCAPSULES" *Diabetologia (1988) 31:54-57*
	26.	Colton, C.K. (1995), "IMPLANTABLE BIOHYBRID ARTIFICIAL ORGANS" *Cell Transplantation 4(4):415-436.*
	27.	Dixit, V. et al., "A MORPHOLOGICAL AND FUNCTIONAL EVALUATION OF TRANSPLANTED ISOLATED ENCAPSULATED HEPATOCYTES FOLLOWING LONG-TERM TRANSPLANTATION IN GUNN RATS" *Biomat. Art. Cells & Immob. Biotech. (1993) 21(2):119-133*
	28.	Gao, E-K et al., "T CELL CONTACT WITH Ia ANTIGENS ON NONHEMOPOIETIC CELLS IN VIVO CAN LEAD TO IMMUNITY RATHER THAN TOLERANCE" *J. Exp. Med. (1991)174:435-446*
	29.	Gilbert, J.C. et al., "CELL TRANSPLANTATION OF GENETICALLY ALTERED CELLS ON BIODEGRADABLE POLYMER SCAFFOLDS IN SYNGENEIC RATS" *Transplantation (1993) 56(2):423-427*
	30.	Hansan, et al., "EVIDENCE THAT LONG-TERM SURVIVAL OF CONCORDANT XENOGRAFTS IS ACHIEVED BY INHIBITION OF ANTISPECIES ANTIBODY PRODUCTION" *Transplantation, (1992) 54:408-413*
	31.	Hill, R.S. et al., "MEMBRANE ENCAPSULATED ISLETS IMPLANTED IN EPIDIDYMAL FAT PADS CORRECT DIABETES IN RATS" *Cell Transplantation (1992) 1(213):132 p. 168*
	32.	Hoffman, D. et al., "TRANSPLANTATION OF A POLYMER-ENCAPSULATED CELL LINE GENETICALLY ENGINEERED TO RELEASE NGF" *Experimental Neurology (1993) 122:100-106*
	33.	Husby, s. et al., "ORAL TOLERANCE IN HUMANS. T CELL BUT NOT B CELL TOLERANCE AFTER ANTIGEN FEEDING" *J. Immunol., (1994)152:4663-4670*
	34.	Knetaman, N.M. et al., "ISOLATION AND CRYOPRESERVATION OF HUMAN PANCREATIC ISLETS" *Transplantation Proceedings (1986) XVIII(1):182-185*
	35.	Lacy, P.E. et al., "MAINTENANCE OF NORMOGLYCEMIA IN DIABETIC MICE BY SUBCUTANEOUS XENOGRAFTS OF ENCAPSULATED ISLETS" *Science (1991) 254:1782-1784*
	36.	Lanza, R.P. et al., "XENOTRANSPLANTATION OF CANINE, BOVINE, AND PORCINE ISLET" *PNAS USA (1991) 88:11100-11104.*
	37.	Lanza, R.P. et al., "TRANSPLANTATION OF ENCAPSULATED CANINE ISLETS INTO SPONTANEOUSLY *Endocrinology (1992), 131(2):637-642*
	38.	Liu, H. et al., "EXPRESSION OF HUMAN FACTOR IX BY MICROENCAPSULATED RECOMBINANT FIBROBLASTS" *Human Gene Therapy (1993) 4:291-301*
	39.	Lum, Z. et al., "PROLONGED REVERSAL OF DIABETIC STATE IN NOD MICE BY XENOGRAFTS OF MICROENCAPSULATED RAT ISLETS" *Diabetes (1991) 40:1511-1516*
	40.	Nossal, G.J.V. "IMMUNOLOGICAL TOLERANCE" In: Fundamental Immunology, Second Edition, edited by W.E. Paul, Raven Press, New York, pp 571-585 (1989)
	41.	Osband, ME et al., "PROBLEMS IN THE INVESTIGATIONAL STUDY AND CLINICAL USE OF CANCER IMMUNOTHERAPY" *Immunological Today, (1990) 11(6):193-195*
	42.	Posselt, A.M. et al., "INDUCTION OF DONOR-SPECIFIC UNRESPONSIVENESS BY INTRATHYMIC ISLET TRANSPLANTATION" *Science (1990) 249:1293-1295*
MB	43.	Posselt, A.M. et al., "INTRATHYMIC ISLET TRANSPLANTATION IN THE SPONTANEOUSLY DIABETIC BB RAT" *Ann. Surg. (1991) 214(4):362-373*

EXAMINER	DATE CONSIDERED 5/11/05

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	FILING DATE September 12, 2003	GROUP 1632

EXAMINER INITIAL		OTHER DOCUMENTS (INCLUDING AUTHOR, TITLE, DATE, PERTINENT PAGES, ETC.)
MB	44.	Ricordi, C. et al., "AUTOMATED METHOD FOR ISOLATION OF HUMAN PANCREATIC ISLETS" *Diabetes (1988) 37:413-420*
	45.	Soon-Shiong, P. et a., "PREVENTION OF CTL AND NK CELL-MEDIATED CYTOTOXICITY BY MICROENCAPSULATION" *Hormone Metab. Res. (1990) 25 (suppl.): 215-219*
	46.	Sullivan, S.J. et al., "BIOHYBRID ARTIFICIAL PANCREAS: LONG-TERM IMPLANTATION STUDIES IN DIABETIC, PANCREATECTOMIZED DOGS" *Science (1991) 252:718-720*
	47.	Tai, I.T. et al., "MICROENCAPSULATION OF RECOMBINANT CELLS: A NEW DELIVERY SYSTEM FOR GENE THERAPY" *FASEB J. (1993) 7:1061-1069*
	48.	Tueveson, G et al., "NEW IMMUNOSUPPRESSANTS: TESTING AND DEVELOPMENT IN ANIMAL MODELS AND THE CLINIC: WITH SPECIAL REFERENCE TO DSG" *Immunological Reviews, (1993) 136:99-109*
	49.	Tresco, P.A. et al., "POLYMER ENCAPSULATED NEUROTRANSMITTER SECRETING CELLS POTENTIAL TREATMENT FOR PARKINSON'S DISEASE" *ASAIO Journal (1992) 38:17-23*
MB	50.	Wong, H. et al., "THE MICROENCAPSULATION OF CELLS WITHIN ALGINATE POLY-L-LYSINE MICROCAPSULES PREPARED WITH THE STANDARD SINGLE STEP DROP TECHNIQUE: HISTOLOGICALLY IDENTIFIED MEMBRANE IMPERFECTIONS AND THE ASSOCIATED GRAFT REJECTION" *Biomat., Art. Cells & Immob. Biotech. (1991) 19(4):675-686*

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Notice of References Cited	Application/Control No. 10/660,924	Applicant(s)/Patent Under Reexamination LATTA, PAUL P.	
	Examiner Michail A. Belyavskyi	Art Unit 1644	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
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	H	US-			
	I	US-			
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	K	US-			
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
X	U	Atkinson et al., Nature, 1999, V.5, pages 601-604
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
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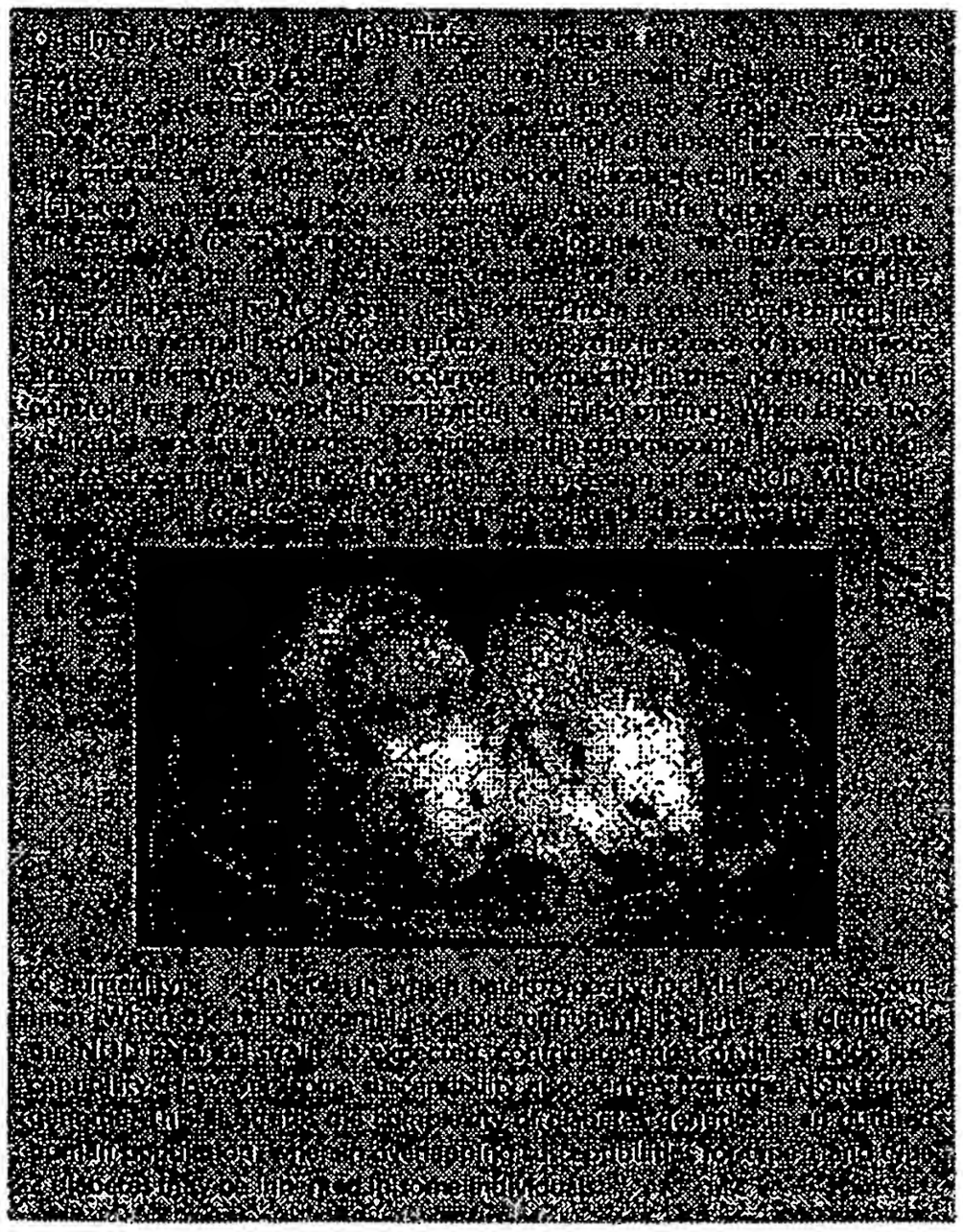
The NOD mouse model of type 1 diabetes: As good as it gets?

MARK A. ATKINSON[1] & EDWARD H. LEITER[2]

Diabetes mellitus in humans is a genetically and clinically heterogeneous group of glucose intolerance syndromes. Type 2 diabetes (also called non-insulin-dependent diabetes mellitus) is the more prevalent clinical form, in which obesity associated with progressively more severe insulin resistance are common predictors of the prediabetic state. Type 1 diabetes (also called insulin-dependent diabetes mellitus, or juvenile diabetes), in contrast, usually has an autoimmune T cell-mediated etiology in which the prediabetic state is characterized by development of autoantibodies against certain proteins expressed by β cells, including insulin. Two rodent models that spontaneously develop type 1 diabetes, the NOD (non-obese diabetic) mouse and the BB (BioBreeding) rat, have allowed detailed exploration of the dysregulated communication between cells of the innate and acquired immune system that underlie the generation and release of pancreatic β cell-reactive T cells.

In the 19 years since the first report of the NOD mouse, this small rodent has eclipsed its 'bigger brother', the BB rat, as the favored model for investigations into the etiopathogenesis of autoimmune, T cell-mediated type 1 diabetes in humans. The reasons for the preferred popularity of the mouse model include a better-defined genome, more monoclonal reagents for the analysis of immune system components and considerably lower maintenance costs. Today, when a candidate autoantigen undergoes evaluation, the effect of a cytokine is tested or a preventative intervention is assessed, NOD mice are often considered 'as good as it gets', short of a study in humans—so much so that other animal models are not always tested nor are important distinctions with the NOD model considered before extrapolations to humans are made.

Indeed, the introduction of NOD mice to diabetes research infused a large sense of optimism, with immunologists initially assuming that the NOD mouse would be a murine 'Rosetta stone' for quickly unraveling the secrets of the etiopathogenesis of type 1 diabetes in humans. This belief found early support through the observation that, as in humans, the major histocompatibility complex (MHC) of the NOD mouse (designated $H2^{g7}$) contributed the main component of susceptibility, and that the MHC class II I-Aβ chain showed the same 'diabetogenic' amino-acid substitution found in the human *DQ*0302* allele associated with high risk for development of type 1 diabetes (a non-aspartic-acid substitution at residue 57 in the β-chain). Rapid cloning of the effector T cell, sequencing of its T-cell receptor (TCR) genes and identification of cognate peptide(s) were predicted to follow. From there, identification of the target β-cell autoantigen and development of blocking peptides or tolerogenic regimens were to be relatively simple matters. Furthermore, it was assumed that identification of non-MHC diabetogenic loci in the NOD mouse would allow for rapid identification of human homologs, thereby allowing accurate prediction of children at high genetic risk for developing type 1 diabetes. Finally, if the target autoantigen in mouse and human β cells were the same, the prevention of type 1 diabetes in the NOD mouse would rapidly be followed by comparable immunologic prediction and, hopefully, the eradication of this disease in humans.



Our understanding of the pathogenic mechanisms underlying type 1 diabetes development in NOD mice is now quite advanced[1]. However, this understanding has been accompanied by the realization that when this mouse is used as a surrogate for humans, genus-specific differences that restrict their interpretation are unavoidable. In addition to certain NOD strain-

Table The 'A-to-Z' of diabetes prevention in the NOD mouse. Therapies include those that either suppress T-cell functions or stimulate the immune system to achieve a more normal immunoregulatory communication between antigen-presenting cells and T cells.

Androgen
Anesthesia
Azathioprine
Anti-B7-1
Bacille Calmette Gue'rin (BCG)
Baculofin
β-1,6;1,3-D-glucan
Anti-β 7 integrin
Blocking peptide of MHC class II
Bone marrow transplantation
Castration
Anti-CD3
Anti-CD4
Anti-CD8
Anti-CD28
Cholera toxin-B subunit
Cold exposure
Anti-complement receptor
Complete Freund's adjuvant
Anti-CTLA-4
Cyclosporin
Cyclosporin A
Dapsone (4,4'-diaminodiphenyl sulfone)
Deflazacort
Dendritic cells from pancreatic lymph node
Deoxysperoguailin
Diazoxide
1,25 dihydroxyl Vitamin D3
Elevated temperature
Encephalomyocarditis virus (ECMV)
Escherichia coli extract
Essential fatty acid-deficient diets
FK506
Gallium nitrate
Glucose (neonatal)
Glutamic acid decarboxylase -intraperitoneal, intrathymic, intravenous, oral
Glutamic acid decarboxylase peptides -intraperitoneal, intrathymic, intravenous, oral
Gonadectomy
Heat shock protein 65
Heat shock protein peptide (p277)
Anti-ICAM-1
Immobilization
Immunoglobulin (IgG2a)
Anti-integrin alpha 4
Inomide
Insulin -intraperitoneal, oral, subcutaneous, nasal
Insulin B chain/B chain amino acids 9–23 -intraperitoneal, oral, subcutaneous, nasal
Insulin-metabolically inactive
Insulin-like growth factor I
Interferon-α
Anti-interferon-γ
Interferon-γ receptor
Interleukin-1
Interleukin-1 receptor
Interleukin-2
Interleukin-2 receptor fusion toxin (DAB480-IL-2)
Interleukin-3
Interleukin-4
Interleukin-10
Interleukin-12 antagonist
Islet cells–intrathymic
Lactate dehydogenase virus (LDH)
Lactobacilus casei
Lazaroid
Linomide
Lithium chloride
Anti-LFA-1
Anti-L-selectin
Lymphocyte choriomeningitis virus (LCMV)
Ahti-lymphocyte serum/lymphotoxin
Lymphocyte vaccination
LZ8
MDL 29311
Melatonin
Anti-MHC class I
Anti-MHC class II
Mixed allogeneic chimerism
Monosodium glutamate
Murine hepatitis virus (MHV)
Mycobacterium
Natural antibodies
Nicotinamide
Nutramigen
OK432
Overcrowding
Pancreatectomy
Pentoxifylline
Pertussigen
Poly [I:C]
Pregestimil diet
Probucol
Prolactin
Rampamycin
Reg protein
Rolipram
Saline (repeated injection)
Semi-purified diet (AIN-76)
Silica
Sodium fusidate
Somatostatin
Non-specific pathogen free conditions
Streptococcal enterotoxins (SEA)
Superantigens
Superoxide dismutase-desferrioxamine
TGF-β
Anti-T-cell receptor
Anti-thy-1
Thymectomy (neonatal)
T-lymphocyte clones
Tolbutamide
Troglitazone
Tumor necrosis factor-α
Tumor necrosis factor-β
Vitamin E
Anti-VLA-4

specific characteristics that distinguish these mice from humans at risk for type 1 diabetes (such as deafness or the absence of C5 complement), important genus-specific features distinguish the murine diabetes as well (such as resistance to ketoacidosis or the absence of the murine homolog of HLA-DR molecules on antigen-presenting cells). Investigators have not always considered that because these mice are so highly inbred, they must be viewed as a single 'case study' in humans. Indeed, the combination of NOD strain-specific features as well as inherent differences between genera may explain why identification of non-MHC diabetogenic loci in mice have not generally been direct guide posts for the identification of homologous loci in outbred humans at risk for type 1 diabetes. As an example, the non-MHC locus (*IDDM2*, chromosome 11) that contributes to increased sibling risk in human studies is associated with a variable-number tandem repeat controlling expression of the closely-linked insulin gene. A susceptibility-conferring homolog has not yet been identified in the NOD mouse, most likely because the mouse genome, unlike the human genome, contains two unlinked insulin genes, both of which are expressed. Nevertheless, certain immunogenetic and immunopathogenic aspects of type 1 diabetes in this mouse 'case study', particularly the main pathogenic contributions made by MHC genes (*Idd1* in NOD mice and *IDDM1* in humans), clearly justify thorough investigation into why MHC-associated deficiencies in immune function allow development of an autoreactive T-cell repertoire.

The etiology of type 1 diabetes in this model is both complex and multifactorial[1–3]. Both $CD4^+$ and $CD8^+$ T cells constitute the effector arm, with underlying functional defects in bone marrow-derived antigen-presenting cells (APCs), including macrophages, dendritic cells and B lymphocytes, shown to be essential components in selection/activation of the autoimmune repertoire. Many $CD4^+$ and $CD8^+$ T-cell lines and clones with diabetogenic potencies against a variety of identified and unidentified antigens have been established from both islets and spleen[4]. If there is a single TCR clonotype distinguishing the 'primordial' diabetogenic T cell, its primacy has not yet been demonstrated. Destruction of β cells apparently entails both necrotic and apoptotic events in response to invasion of the islets by leukocytes (insulitis)[2]. There are large numbers of leukocytes in the insulitic infiltrates of NOD mice, almost suggesting lymph node formation around islets. Insulitis in a human acute-onset diabetic is very different from that in NOD islets (Fig.). One of the strain-specific peculiarities of NOD mice is the accumulation of many T cells in peripheral lymphoid organs, pancreas and submandibular salivary glands. This T-lymphocyte accumulation possibly reflects low IL-2 levels and the resistance of thymocytes and peripheral T cells to the induction of apoptosis.

Although they are important in improving our understanding of the cause(s) and pathogenesis of this disease, these immunologic features are also vital for this model to serve as a tool in identifying potential therapeutic modalities for the prevention of human type 1 diabetes. As of early 1999, more than 125 individual methods reporting the prevention or delay of

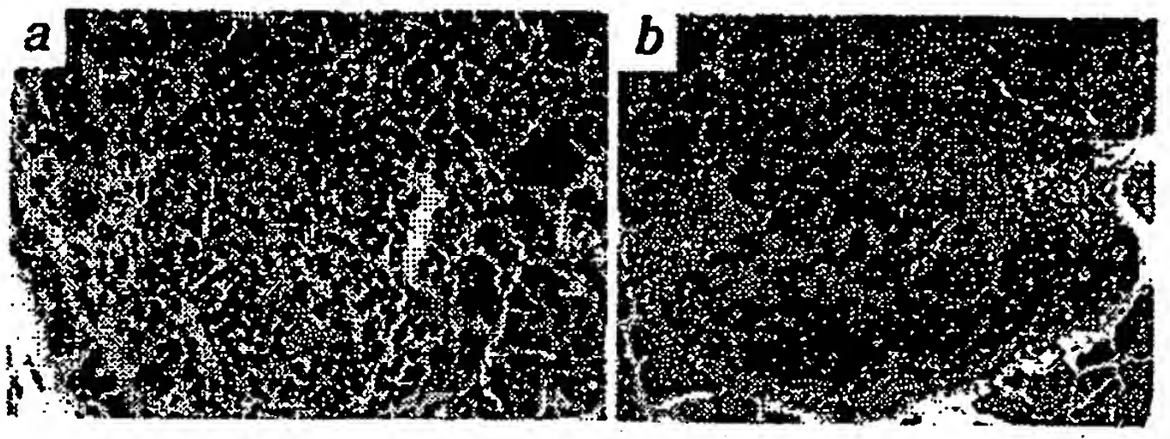


Fig. 1 Insulitis in humans and NOD mouse pancreas. *a*, Pancreas section with heavy leukocyte infiltration (insulitis) in a human islet. The donor died a few days after acute onset of type 1 diabetes. (photo courtesy of Massimo and Trucco, Department of Pediatrics, University of Pittsburgh). Hematoxylin and eosin stain. *b*, Pancreas of a prediabetic 12-week-old NOD/Lt mouse, showing the unusually heavy accumulation of leukocytes adjacent to and infiltrating the islets. The pancreatic β cells with in the islet have been stained purple with aldehyde fuchsin.

type 1 diabetes in NOD mice have been identified (Table). These interventions can be grouped into two general categories: treatments that actually suppress T-cell function and treatments that modulate immune communication, often by actually stimulating certain immune functions. Although this list is long, we limited reference to studies monitoring spontaneous type 1 diabetes and excluded reports whose practical relevance to human disease is unclear. (For example, introduction of genetically-disrupted genes whose normal counterparts are required for (auto)antigen presentation, for T-cell effector functions, or for rearrangement of functional T-cell receptors.) The ease by which immunomodulation diverts the immune system in these mice is best understood by considering the effect of their exposure to extrinsic microbial pathogens. The inbreeding of NOD mice has genetically fixed a number of immunodeficiencies that, in aggregate, are reflected by impaired communication between APC and T cells. NOD macrophages have an impaired ability to activate regulatory T cells in an autologous mixed lymphocyte reaction. Comparable impairment in dendritic cell function has been seen in patients with recent-onset type 1 diabetes[4]. The NOD immunodeficiences are partially correctable in a natural environment, in which a full range of microbial and viral antigens would be encountered. It is only when NOD mice are maintained in stringent specific pathogen-free conditions that full disease penetrance of the underlying genetic type 1 diabetes susceptibility will be seen in both sexes. Thus, the NOD model is one in which paradoxical immunostimulation effected by a variety of treatments ameliorates the weak communication between the innate and adaptive immune system components and thereby restores more normal control over autoreactive T cells. Unfortunately, in a genetically heterogeneous human population containing individuals at high risk for type 1 diabetes development, there is little evidence that many of them would have a comparable set of immune deficiencies that prove as malleable. At the same time, the observation that 'cleaning up' the extrinsic environment of the NOD mouse sets the stage for activation of autoimmune T cells raises the question of whether a hypersanitized rearing environment for human infants might predispose children with autoimmune-permissive HLA haplotypes to higher risk for eventual penetrance of autoimmune diseases.

In NOD mice, type 1 diabetes development is well-choreographed when all the relevant environmental factors (pathogen status, diet and so on) are held constant. Specific 'time windows' can be defined in which an immunomodulator can be either protective or destructive. In contrast, the natural history of type 1 diabetes in humans is such that the age of disease onset is extremely broad; symptoms occur at any time from the first years of life to well beyond 50 years of age. Although it is a potential limitation in comparing the natural history of type 1 diabetes in NOD mice with humans, this factor can be turned into a strength through proper matching of therapeutic agents to the appropriate time for human intervention. Intervention studies in NOD mice can be designed in which therapeutic regimens are initiated at birth, at a presymptomatic stage before the occurrence of insulitis (that is, less than three weeks postpartum); before the onset of symptomatic disease (that is, four to eight weeks postpartum) at a time when considerable numbers of β cells are still intact; or at the diagnosis of type 1 diabetes, when β-cell damage has accumulated to the extent of overt hyperglycemia. However, studies analyzing therapeutic agents aimed at preventing type 1 diabetes in NOD mice must be carefully assessed for their functional as well as their practical applicability to therapeutic intervention in human disease. This has not always been considered. For example, agents used in NOD mice from birth (a time without β-cell destruction) may not be applicable to treatment of humans identified immediately before the onset of type 1 diabetes (when substantial β-cell destruction has occurred).

It is clear that the genus-unique and strain-specific aspects of diabetogenesis in NOD mice must be fully understood and appreciated if we are to know which therapeutic protocols are reasonable to extrapolate to humans and which are not. In addition, intervention protocols effective in preventing type 1 diabetes in NOD mice should be studied in as many animal models as are available. The rationale for implementing insulin prophylaxis therapy to prevent type 1 diabetes in humans was based on the observation that insulin treatment of both prediabetic NOD mice and BB rats retarded onset and reduced disease frequency. However, one may question whether another candidate β-cell autoantigen, glutamic acid decarboxylase (GAD), will provide similarly promising results. Isoforms of this enzyme are relatively easy to detect in human and rat β cells. In contrast, GAD protein is present at considerably lower concentrations in NOD islets and what little GAD67 isoform can be detected may not all derive from β cells. Nevertheless, NOD mice can be deviated from diabetes by early treatment with recombinant GAD protein or peptides. In contrast, GAD autoimmunity does not seem to be a factor in BB rats, emphasizing the point that many models should be evaluated before extrapolations to humans are attempted. With such an appreciation comes the realization that it is also essential to extend mechanistic studies in the NOD mouse to the rat models of spontaneous and induced type 1 diabetes. In addition to the lymphopenic BB/Wor diabetes-prone rat that develops type 1 diabetes spontaneously, type 1 diabetes can be induced in the non-lymphopenic BB/WOR diabetes-resistant substrain and in other strains by immunomodulation coupled with exposure to a parvovirus, Kilham rat virus[5]. Thus, it is the rat and not the mouse model that the investigator should first consider if the object is to an-

alyze the potential of environmental viral pathogens to serve as diabetogenic triggers. New rat models spontaneously developing type 1 diabetes, such as the non-lymphopenic Komeda diabetes-prone (KDP) rat[6], should provide additional pathogenic insights as they become available.

At a minimum, investigations of NOD mice have enhanced our appreciation of the etiologic complexity of type 1 diabetes in humans and provided an example of how promising results obtained in an animal model can be translated into human clinical trials. However, exploitation of the peculiarities of the NOD genome for clinical research is yet to be fully realized. Specifically, further investigation of NOD mice should advance our understanding of the genetic and pathophysiologic basis for other complex pathologies (such as thyroiditis, lupus, sialoadenitis, deafness and inflammatory bowel disease). The strain's robust breeding performance, its extensively characterized genome and the availability of type 1 diabetes-resistant MHC-congenic stocks render NOD mice ideal for outcross with other inbred strains carrying gene mutations for physical mapping/positional cloning analyses. Deficiencies in immunoregulation (such as dysfunctional NK cells or the absence of hemolytic complement) make NOD mice congenic for additional immunodeficiency genes (*scid* and *rag*) ideal hosts for carrying human cells, especially when they are further modified by the transgenic insertion of human genes and/or simultaneous elimination of select murine genes. This technology is providing stocks suitable for analysis of the growth, development and survival of human hematopoietic cells. The immunocompromised NOD mice should also prove useful for the study of human infectious diseases, including AIDS, filariasis and malaria. Gene targeting technology and the ability to produce tissue-specific knockouts of genes are allowing dissection of pathogenic pathways not easily amenable to study in humans.

In sum, criticisms of the inbred nature and controlled housing environment, the ability to change natural physiology through genetic manipulation and the relative ease for disease prevention have caused some to question whether the model is 'as good as it gets'. It is clear that the course of type 1 diabetes development in randomly breeding humans will not be as easily deviated as it is in highly inbred rodent models in which genetic risk is a constant such that interventions can be initiated at very early stages of pathogenesis. Thus, no investigator should assume that the available mouse and rat models spontaneously developing type 1 diabetes represent complete surrogates for humans. However, the fact that diabetes in these rodents develops spontaneously rather than in response to investigator-induced gene targetings allows acquisition of essential insights into the interactions between genes and environment that together trigger a complex disease.

1. Leiter, E.H. & Atkinson, M.A. NOD mice and related strains: Research applications in diabetes, AIDS, cancer and other diseases in *Medical Intelligence Unit* (R.G. Landes, Austin, 1998).
2. Delovitch, T.L. & Singh, B. The nonobese diabetic mouse as a model of autoimmune diabetes - immune dysregulation gets the NOD. *Immunity* 7, 727–738 (1997).
3. Rabinovich, A. An update of cytokines in the pathogenesis of insulin dependent diabetes mellitus. *Diabetes Metab. Rev.* 14, 129–151 (1998).
4. Takahashi, K., Honeyman, M.C. & Harrison, L.C. Impaired yield, phenotype, and function of monocyte-derived dendritic cells in humans at risk for insulin dependent diabetes. *J. Immunol.* 161, 2629–2635 (1998).
5. Ellerman, K.E., Richards, C.A., Guberski, D.L., Shek, W.R. & Like, A.A. Kilham rat virus triggers T-cell-dependent autoimmune diabetes in multiple strains of rats. *Diabetes* 45, 557–562 (1996).
6. Komeda, K. *et al.* Establishment of 2 substrains, diabetes-prone and nondiabetic, from Long-Evans Tokushima-Lean (LETL) rats. *J. Endocrinol.* 45, 737–744 (1998).

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